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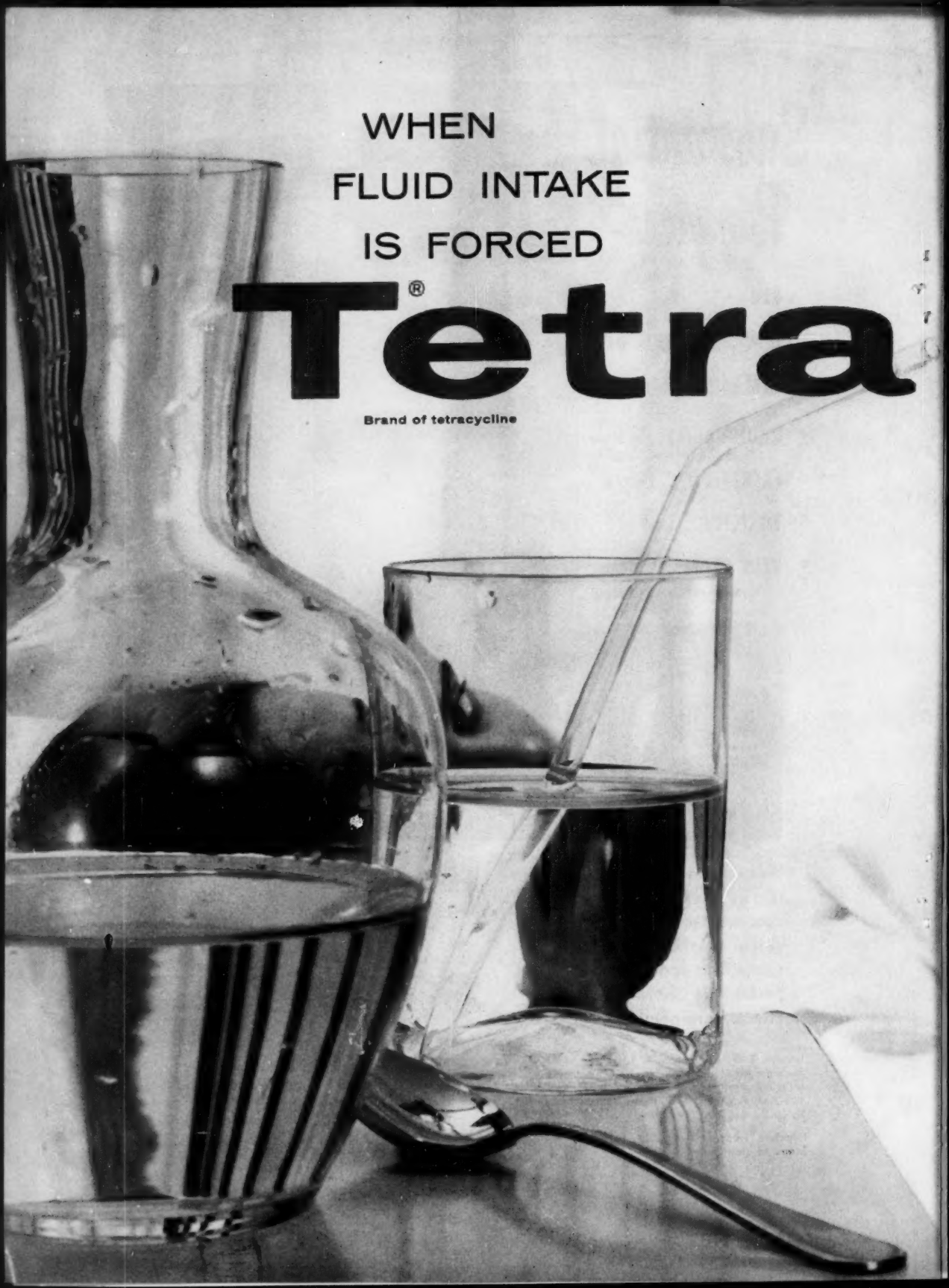
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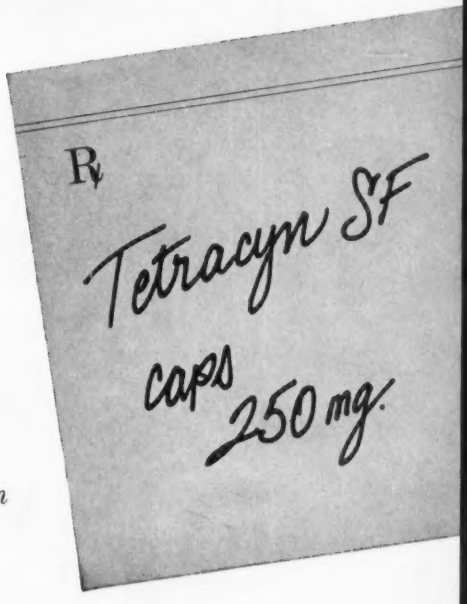

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1. Dumas, K. J.; Carlozzi, M., and Wright, W. A.: Antibiotic Med. 1:296 (May) 1955.
2. Prigot, A.: Ann. New York Acad. Sc., in press.
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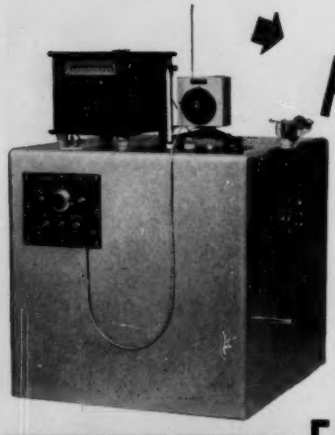

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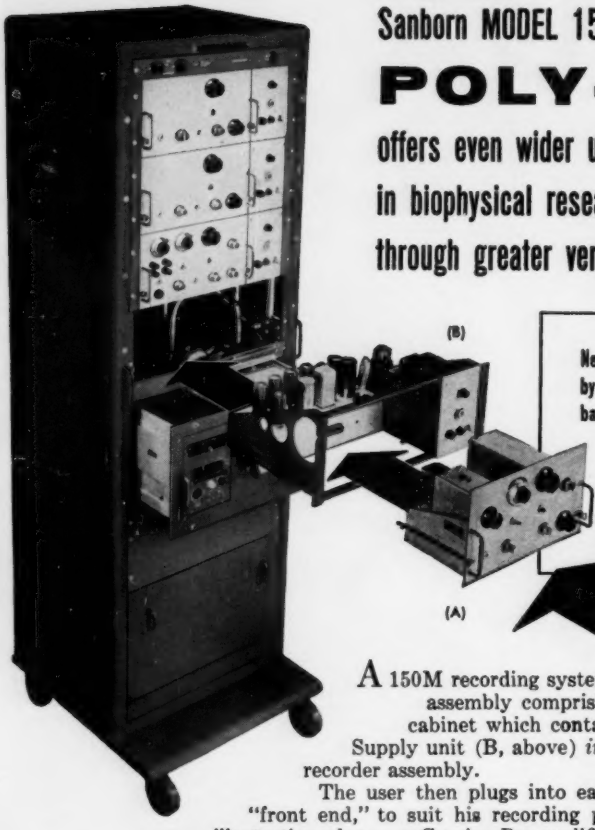

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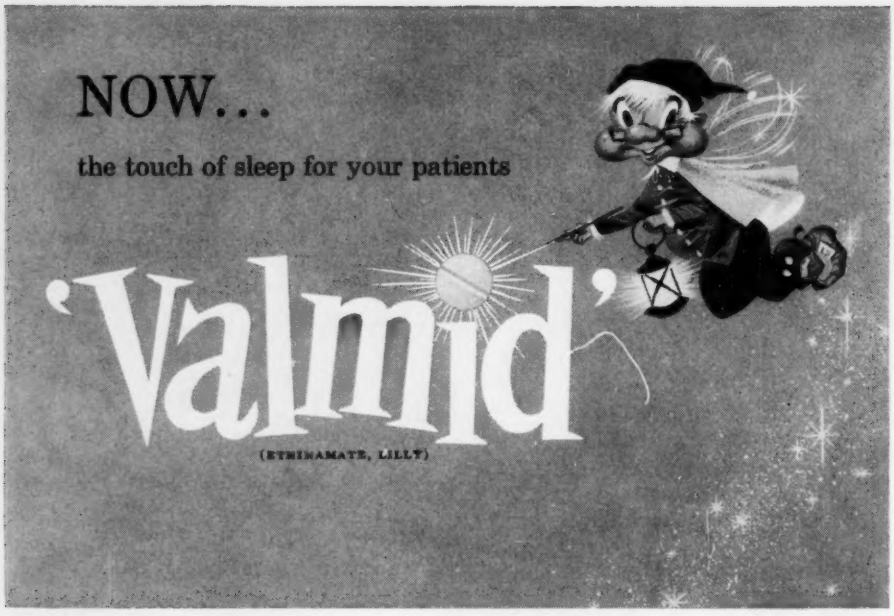

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THE DISTRIBUTION AND CHEMICAL COMPOSITION OF ULTRACENTRIFUGALLY SEPARATED LIPOPROTEINS IN HUMAN SERUM

By RICHARD J. HAVEL, HOWARD A. EDER, AND JOSEPH H. BRAGDON

(From the Laboratory of Metabolism, National Heart Institute, National Institutes of Health, Department of Health, Education, and Welfare, Bethesda, Md.)

(Submitted for publication January 17, 1955; accepted April 13, 1955)

In the past few years several methods have been developed for the analysis of serum lipoproteins. Lindgren, Elliott, and Gofman (1) have utilized the relatively low density of the lipoproteins to separate them from the other serum proteins by ultracentrifugal flotation. Quantitation was subsequently performed by refractometric methods in the analytical ultracentrifuge. Separations of lipoproteins have also been made by Cohn fractionation in cold ethanol, and the quantities of lipoprotein have been estimated from the lipid content of the fractions (2, 3). Widely used at the present time is the method of zone electrophoresis with quantitation either by staining (4) or by chemical analysis of eluates from the supporting medium (5, 6).

Each of these methods has serious limitations. Analytical ultracentrifugal techniques (7, 8) require the possession of expensive equipment. The quantitation of data is subject to considerable error and gives no information regarding the chemical composition of the lipoproteins. Cohn fractionation requires facilities for operation at $-5°$ C. It permits accurate determination of the lipid components of the alpha and beta lipoproteins, but with this technique it is impossible to subfractionate these groups. With certain abnormal sera the method is unreliable (9). Determination of electrophoretically separated fractions by staining techniques or by chemical analysis of eluates is subject to appreciable error. Both Cohn fractionation and electrophoretic techniques fail to separate lipoproteins from other serum proteins, thus making impossible the study of the protein moiety.

The combination of preparative ultracentrifugation with chemical analysis of the separated fractions would seem to be a procedure by which both the distribution and composition of lipoproteins could be determined simply and accurately. Such a procedure was described by Turner, Snavely, Goldwater, Randolph, Sprague, and Unglaub (10). Under their conditions of ultracentrifugation, however, separation of discrete lipoproteins did not occur and their results are difficult to interpret. In the present study, lipoproteins have been separated from serum by repeated ultracentrifugations after progressively raising the solvent density. The separated fractions have been analyzed for total cholesterol, lipid phosphorus, and protein. The method has proved reliable and is, in our opinion, the simplest available procedure for the accurate quantitation of serum lipoproteins.

METHODS

The density of serum was raised by the addition of concentrated salt solution. The stock salt solution contained 153.0 gm. sodium chloride and 354.0 gm. potassium bromide per liter (density: 1.346). Solutions of lower density were prepared by dilution of the stock solution with 0.15 M sodium chloride solution (density: 1.005) according to the formula:

$$A \times 1.005 + B \times 1.346 = (A + B)X,$$

where A is the volume of 0.15 M saline, B the volume of stock solution, and X the desired density of the mixture. The density of serum or of infranates was raised to the desired non-protein solvent density by the addition of a salt solution according to a similar formula:

$$A \times Y + B \times Z = (A + B)X,$$

TABLE I

Preparation of samples of known solvent density for preparative ultracentrifugation

Serum		Salt Solution		Final
Solvent Density	Volume (ml)	Density	Volume (ml)	Solvent Density of Mixture
1.006	10	1.346	2	1.063
1.006	10	1.085	2	1.019
1.019	9	1.200	3	1.064

TABLE II

Distribution and composition of lipoproteins in fractions of human serum separated at density 1.063

	Age	Serum			Density <1.063			Density >1.063		
		Total Chol. (mg/100 ml)	Phospho-lipid (mg/100 ml)	TC* PL	Total Chol. (mg/100 ml)	Phospho-lipid (mg/100 ml)	TC PL	Total Chol. (mg/100 ml)	Phospho-lipid (mg/100 ml)	TC PL
	Females									
	20	125	165	.76	80	63	1.27	48	105	.46
	23	141	183	.77	99	86	1.15	34	92	.37
	23	142	197	.72	78	56	1.39	57	132	.43
	25	154	200	.77	106	83	1.28	49	118	.42
	24	155	204	.76	84	65	1.29	67	142	.47
	23	159	222	.72	97	86	1.13	55	135	.41
	22	166	226	.73	92	79	1.16	67	144	.47
	24	166	200	.83	104	75	1.31	65	125	.52
	26	170	226	.75	118	99	1.19	50	124	.40
	23	171	207	.83	129	104	1.24	40	112	.36
	29	174	214	.81	104	75	1.31	66	132	.50
	29	176	235	.75	108	83	1.30	65	137	.47
	29	178	223	.80	122	92	1.33	50	124	.40
	27	182	230	.79	150	117	1.28	36	116	.31
	26	188	234	.80	128	96	1.33	57	127	.45
	25	190	232	.82	124	94	1.32	53	130	.41
	25	191	253	.75	128	111	1.15	56	150	.37
	22	193	244	.78	134	117	1.15	53	135	.39
	26	205	271	.76	120	96	1.25	73	163	.45
	23	205	284	.72	134	111	1.21	66	173	.38
	22	208	247	.84	122	90	1.36	84	162	.52
	29	226	281	.80	154	119	1.29	61	149	.41
	27	234	297	.79	161	126	1.28	68	159	.43
Mean	25.0	178	230	.78	116	92	1.26	57	134	.43
Std. Dev.	2.6	27	33	.04	23	19	.08	12	20	.05
	Males									
	21	118	155	.76	75	60	1.25	39	93	.42
	26	144	204	.71	105	98	1.07	31	98	.32
	26	154	208	.74	110	96	1.15	47	112	.42
	23	155	189	.82	112	86	1.30	47	108	.36
	26	156	196	.80	102	78	1.31	58	122	.47
	25	158	192	.82	110	83	1.33	45	113	.40
	29	162	229	.71	113	91	1.24	52	129	.40
	28	166	211	.78	120	97	1.24	42	114	.37
	26	169	230	.73	118	101	1.17	48	125	.38
	26	171	215	.80	128	107	1.20	38	102	.37
	24	174	223	.78	133	108	1.23	40	113	.35
	27	175	228	.77	122	108	1.13	47	110	.43
	28	177	225	.79	122	93	1.31	56	135	.41
	23	177	231	.77	132	100	1.32	54	125	.43
	20	181	225	.80	145	114	1.27	39	117	.33
	28	187	259	.72	145	127	1.14	43	133	.32
	26	195	227	.86	145	108	1.34	46	117	.39
	29	201	223	.90	161	117	1.38	52	112	.46
	29	204	228	.91	149	117	1.27	45	107	.35
	28	210	252	.83	157	119	1.32	47	124	.38
Mean	25.6	172	218	.79	125	100	1.25	46	116	.39
Std. Dev.	3.0	22	22	.06	21	16	.08	7	11	.04
p**		.5>p>.4	.2>p>.1		.1>p>.05	.2>p>.1		p<.01	p<.01	

* Ratio total cholesterol to phospholipid.

† Std. Dev. $= \sqrt{\frac{\epsilon(x - \bar{x})^2}{n - 1}}$.

** Probability that difference between mean values for males and females is due to chance.

where A is the volume of serum or serum infranate, B the volume of salt solution, Y the non-protein solvent density of the sample whose density is to be raised (for serum, this is 1.006), Z the density of the salt solution, and X the non-protein solvent density of the mixture. In Table I are listed the dilutions used in these experiments.

Ultracentrifugation was carried out in the 40 rotor of a Spinco preparative ultracentrifuge (Model L). Eight to 10 ml. of serum were delivered into a capped, plastic tube from a calibrated syringe. The appropriate volume of salt solution was added and the tube filled with a small amount of salt solution of the same density as the mixture. After centrifugation at 105,000 × G. for 20 to 22 hours at 12 to 15° C., lipoproteins of less than solvent density were concentrated in a layer at the top of the tube. Beneath this was a clear, colorless region occupying approximately one-fourth of the length of the tube; the remainder of the serum was stratified below the clear zone. The tube was placed in a tube-slicing device similar to that described by Randolph and Ryan (11) and sliced in the middle of the clear zone. The supernatant contents were transferred quantitatively with washings of 0.15 M sodium chloride solution to a 5 ml. volumetric flask. The infranate was similarly transferred to a 10 ml. volumetric flask with washings of salt solution of equivalent density. If further separation was to be carried out, additional salt solution was added to an aliquot of the infranate and centrifugation was repeated. In this way it is possible to separate several lipoprotein fractions from the same serum sample.[1]

Aliquots of the supernate(s), final infranate, and the original serum were extracted in ethanol-acetone (1:1) and analyzed for total cholesterol by the Sperry-Schoenheimer method (12) and lipid phosphorus by a modification of the method of Stewart and Hendry (13). Lipid phosphorus was converted to phospholipid by the factor 25. In some instances protein content of the ethanol-acetone insoluble material was determined by a modification of the biuret method (14). The insoluble material was washed twice in ethanol-acetone, dried, and dissolved in an amount of sodium hydroxide solution equivalent to that contained in the usual biuret reagent. Biuret reagent without alkali was then added to the alkaline protein solution for color development.

Subjects

Forty-three men and women aged 20 to 30 were studied. They were physicians, nurses, and laboratory

[1] Recent studies indicate that the 40.3 preparative rotor is more efficient for these purposes. It requires a small sample of serum (4 to 5 ml.) and the time of centrifugation can be reduced to 16 hours.

TABLE III

Distribution and composition of lipoproteins in fractions of human serum separated at densities 1.019 and 1.063

	Age	Serum			Density <1.019			Density 1.019-1.063			Density >1.063		
		Total Chol. (mg/100 ml)	Phospho-lipid (mg/100 ml)	TC PL	Total Chol. (mg/100 ml)	Phospho-lipid (mg/100 ml)	TC PL	Total Chol. (mg/100 ml)	Phospho-lipid (mg/100 ml)	TC PL	Total Chol. (mg/100 ml)	Phospho-lipid (mg/100 ml)	TC PL
Females													
	23	141	183	.77	23	27	.85	76	59	1.29	34	92	.37
	23	159	222	.72	19	26	.73	78	60	1.30	55	135	.41
	26	170	226	.75	21	25	.84	97	74	1.31	50	124	.40
	25	171	207	.83	18	26	.69	111	78	1.42	40	112	.36
	29	178	223	.80	18	18	1.00	104	74	1.41	50	124	.40
	25	190	232	.82	8	12	.67	116	82	1.41	53	130	.41
	25	191	253	.75	16	27	.59	112	84	1.33	56	150	.37
	23	205	284	.72	15	22	.68	119	89	1.34	66	173	.38
	29	226	281	.80	38	38	1.00	116	81	1.43	61	149	.41
	27	234	262	.89	13	18	.72	148	98	1.51	72	146	.49
Mean	25.5	187	237	.79	19	24	.78	108	78	1.38	54	134	.40
Std. Dev.	2.3	29	32	.05	8	7	.12	19	12	.06	11	23	.04
Males													
	21	118	155	.76	14	15	.93	61	45	1.35	39	93	.42
	20	144	204	.71	35	40	.88	70	58	1.21	31	98	.32
	25	158	192	.82	18	21	.86	92	62	1.48	45	113	.40
	28	166	211	.79	28	33	.85	92	64	1.44	42	114	.37
	26	169	230	.73	17	22	.77	101	79	1.28	48	125	.38
	26	171	215	.80	25	36	.69	103	71	1.45	38	102	.37
	27	175	228	.77	36	42	.86	86	66	1.30	47	110	.43
	26	195	227	.86	32	39	.82	113	69	1.63	46	117	.39
	29	204	232	.88	21	29	.72	133	89	1.49	43	114	.38
	28	210	252	.83	35	36	.97	122	83	1.47	47	124	.38
Mean	25.6	171	215	.80	26	31	.84	97	69	1.41	43	111	.38
Std. Dev.	3.1	28	27	.05	8	9	.09	22	13	.13	5	11	.06
p		.3>p>.2	.2>p>.1		.1>p>.05	.1>p>.05		.3>p>.2	.2>p>.1		.02>p>.01	.02>p>.01	

personnel. Individuals with known disease were excluded from this group. In addition, sera from several individuals with known disorders of lipid metabolism as well as a variety of animal sera were examined. Blood samples from humans were drawn following an overnight fast or a fat-free breakfast. Pooled serum obtained from the American Red Cross blood donor service was used for detailed analysis of high density lipoproteins.

RESULTS

1. *Young adult humans*

In Table II it can be seen that ultracentrifugation of serum at density 1.063 separates the lipoproteins into two components differing markedly in composition. Roughly 70 per cent of the total cholesterol and 50 per cent of the phospholipid are in the fraction having a density less than 1.063. When analyzed by paper electrophoresis the low density ($D < 1.063$) lipoproteins have the mobility of beta lipoprotein and the high density ($D > 1.063$) lipoproteins that of alpha lipoprotein. Although the concentrations of serum total cholesterol and phospholipid are similar in young men and women in this series, men have more lipid in the $D < 1.063$ fraction, and women more in the $D > 1.063$ fraction. Subfractionation of the $D < 1.063$ fraction reveals that there is more lipid in the lowest density fraction ($D < 1.019$) in males (Table III). This occurs despite the fact that in this smaller group the serum total cholesterol and phospholipid concentrations are appreciably higher in the females. This difference is also apparent to some degree in the intermediate fraction (D 1.019–1.063). The ratio of total cholesterol to phospholipid in the fractions is similar in both sexes. It can be seen that the ratio in the $D < 1.019$ fraction is similar to that of whole serum, whereas that in the D 1.019–1.063 fraction is higher and that in the $D > 1.063$ fraction is much lower.

For both fractionation procedures in these groups recovery of total cholesterol and phospholipid in the fractions ranged from 94 to 106 per cent of the serum value; recovery averaged 98 per cent for total cholesterol and 99 per cent for phospholipid. In order to test the reproducibility of the method, a sample of pooled human serum was fractionated in triplicate using the second fractionation procedure. Duplicate analyses for total cholesterol and phospholipid and single analyses

TABLE IV

Distribution and composition of lipoproteins in various disease states in humans

Age and Sex	Diagnosis	Serum			Density <1.019				Density 1.019-1.063				Density >1.063		
		Total Chol. (mg/100 ml)	Phospholipid (mg/100 ml)	TC/PL	Total Chol.	Phospholipid (mg/100 ml)	Protein	TC/PL	Total Chol.	Phospholipid (mg/100 ml)	Protein	TC/PL	Total Chol. (mg/100 ml)	Phospholipid (mg/100 ml)	TC/PL
20-30 F & M	Mean values for young adults	179	226	.79	23	28	17	.82	103	74	60	1.39	49	123	.40
36 M	Post-myocardial infarction	214	268	.80	43	51	31	.84	119	92	83	1.29	42	110	.38
37 M	Idiopathic hyperlipemia	272	364	.75	196	200	83	.98	70	63	70	1.11	20	113	.17
38 F	Xanthoma tendinosum	344	317	1.09	43	43	23	1.0	233	157	124	1.48	50	110	.45
29 F	Nephrotic syndrome	477	544	.88	255	257	109	.99	137	109	110	1.26	78	155	.50
2 F	Nephrotic syndrome	1058	1064	.99	907	791	286	1.14	90	87	90	1.03	82	132	.62
26 M	Infectious hepatitis	169	328	.52	30	59	32	.51	132	208	112	.63	9	56	.16
35 M	Obstructive jaundice	204	312	.65	117	153	87	.76	87	88	68	.76	13	66	.20
50 F	Primary biliary cirrhosis	340	776	.41	48	96	15	.50	276	586	90	.47	13	77	.17
57 F	Primary biliary cirrhosis	801	1595	.50	79	122	62	.65	696	1432	261	.49	11	81	.14

TABLE V

Distribution and composition of lipoproteins in various mammals

Species	Serum			Density <1.019				Density 1.019-1.063				Density >1.063		
	Total Chol.	Phospho-lipid	TC/PL	Total Chol.	Phospho-lipid	Pro-tein	TC/PL	Total Chol.	Phospho-lipid	Pro-tein	TC/PL	Total Chol.	Phospho-lipid	TC/PL
	(mg/100 ml)			(mg/100 ml)				(mg/100 ml)				(mg/100 ml)		
Mean values for young adult humans	179	226	.79	23	28	17	.82	103	74	60	1.39	49	123	.40
Rabbit	41	93	.44	15	18	14	.83	9	11	12	.83	17	69	.25
Rat	50	114	.44	6	17	12	.35	10	13	17	.77	31	78	.40
Pig	106	161	.66	8	12	10	.67	51	37	36	1.4	41	102	.40
Hamster	121	269	.45	25	44	21	.57	29	34	22	.85	61	176	.35
Dog	150	362	.41	2	10	9	.20	10	19	17	.53	127	325	.39
Monkey	173	295	.59	17	20	13	.85	64	64	52	1.0	80	206	.39

for protein were carried out on the fractions. For total cholesterol the mean values for the separately centrifuged fractions differed by no more than ± 3 per cent; this difference was of the same order of magnitude as the maximum difference between duplicate chemical analyses. For phospholipid the fractions differed by no more than ± 2 per cent; the difference between the duplicates was no more than ± 1 per cent. Protein content of the separately centrifuged fractions differed by no more than ± 1 per cent.

2. *Patients with hyperlipoproteinemia*

In Table IV are shown examples of lipoprotein distribution and composition in several diseases associated with hyperlipoproteinemia. In the first group are patients with atherosclerosis or diseases which predispose to it; in the second group are patients with liver disease.

In the first group the concentration of the $D < 1.063$ fraction is increased in all patients. This increase may be entirely in the D 1.019–1.063 fraction, in the $D < 1.019$ fraction, or in both. The $D > 1.063$ fraction shows no consistent pattern of variation. Thus, in the patient with idiopathic hyperlipemia the concentration increment is entirely in the $D < 1.019$ fraction, and the other fractions are actually decreased. In contrast the patient with xanthoma tendinosum shows a marked increase in the D 1.019–1.063 fraction, very slight increase in the $D < 1.019$ fraction, and normal $D > 1.063$ fraction. Alterations in chemical composition of the fractions are also apparent. The proportion of protein to cholesterol is often altered in the two fractions in which it was measured. Cholesterol-phospholipid ratios differing from the normal are found in all fractions.

In the patients with liver disease the most consistent finding is a decrease in the lipid of the $D > 1.063$ fraction. The low density fractions are uniformly increased. In these patients the separation of the two low density lipoprotein fractions was incomplete as judged by the absence of the colorless solution beneath the top layer. Hence it was necessary to choose the point of separation arbitrarily. Noteworthy are the alterations in composition of the fractions. The cholesterol-phospholipid ratios are uniformly reduced and the proportion of protein to cholesterol also varies considerably.

3. *Animals*

From Table V it can be seen that animals have a higher proportion of their lipid in the $D > 1.063$ fraction than do humans. In some the absolute quantity of this fraction is also considerably higher than in humans. In the dog the serum total cholesterol concentration is only slightly lower than that seen in humans but almost all of it is contained in the $D > 1.063$ lipoproteins. In this fraction the cholesterol-phospholipid ratio is fairly constant in the various species, but it varies considerably in the other fractions. The relative

proportion of protein in the lower density fractions also varies considerably. Of all the mammals studied only the pig closely approximates the human in distribution and composition of lipoproteins.

4. *Subfractionation of the D > 1.063 fraction*

To a D > 1.063 fraction prepared from pooled human serum was added a quantity of solid potassium bromide sufficient to bring the non-protein solvent density to 1.125. This solution was then subjected to ultracentrifugation in the J rotor of the Spinco Model E ultracentrifuge at 173,000 × G. for 18 hours. The supernate was removed in the usual fashion. The solvent density of the infranate was then raised to 1.21 by further addition of potassium bromide and the centrifugation and separation repeated. The chemical composition of the three fractions obtained is shown in Table VI.

TABLE VI

Distribution and composition of human serum lipoproteins of density >1.063

	Total	Density 1.063-1.125	Density 1.125-1.21	Density >1.21
	mg/100 ml native serum			
Total cholesterol	35	14	20	<2
Phospholipid	101	28	43	27
Protein	--	39	87	--
$\frac{TC}{PL}$	.35	.50	.47	<.1
$\frac{TC}{Protein}$	--	.36	.23	--

It is evident that cholesterol and lipid phosphorus are present in the 2 fractions of D < 1.21. Their chemical composition differs only in the higher protein content of the D 1.125–1.21 fraction. The D > 1.21 fraction contains lipid phosphorus but practically no cholesterol. Ethanol-acetone soluble phosphorus has been found repeatedly in 1.21 infranates, both from pooled serum and fresh serum, whether centrifuged initially at density 1.21 or following preliminary flotation at lower densities. Further, no difference has been observed whether flotation was carried out in the 40 rotor for 48 hours, the 40.3 rotor for 23 hours, or the J rotor for 18 hours. In six young adults the 1.21 density infranate contained 15 to 27 mg. per 100 ml. of the serum phospholipids, and in eight hyperlipoproteinemic individuals, 13 to 25 mg. per 100 ml. In all instances, total cholesterol concentration invariably has been less than 3 mg. per 100 ml. of serum by the method of Abell, Levy, Brodie, and Kendall (15) and usually zero by the Sperry-Schoenheimer method. Thus, it may be that this Liebermann-Burchard reactive substance is not cholesterol. This fraction has been further characterized in the following ways. The lipid phosphorus in 1.21 density infranates fails to float at density 1.30 following centrifugation at 173,000 × G. for 18 hours. It is not dialyzable against 0.15 M sodium chloride and is precipitated with the serum proteins by zinc hydroxide. In starch electrophoresis the bulk of the lipid phosphorus migrates with the alpha$_1$-albumin fraction, the remainder trailing with the other globulin fractions.[2]

TABLE VII

Comparison of lipoprotein values in young adults obtained by various methods

Method of Analysis	Density <1.019		Density 1.019-1.063		Density >1.063	
	Male	Female	Male	Female	Male	Female
	mg/100 ml serum or plasma					
Present Method *	170	130	320	360	340	430
Analytical Ultracentrifuge at Density 1.063 *(17)	179	107	303	264	---	---
Analytical Ultracentrifuge at Density 1.21 ‡(19)	---	---	180	160	154	183
Cohn Fractionation ‡ @ (3)	---	---	---	---	360	460

* Age group 20–30.
‡ Age group 18–35.
@ Bloor cholesterol multiplied by 0.9 prior to conversion to lipoprotein.

DISCUSSION

In Table VII serum lipoprotein concentrations obtained by this method are compared with values obtained by other methods. For our data and for those of Russ, Eder, and Barr (3), lipoprotein concentrations have been calculated from the cholesterol values by utilizing the known cholesterol contents of lipoprotein fractions isolated from pooled human serum. These values are: D < 1.019, 15 per cent; D 1.019–1.063, 30 per cent; and D 1.063–1.21, 12.5 per cent (16). The

[2] We are indebted to Dr. Henry G. Kunkel of the Rockefeller Institute for Medical Research for performing this analysis.

D < 1.019 fraction corresponds roughly to the Gofman S_f 12-400 class, the D 1.019–1.063 fraction corresponds to the Gofman S_f 0-12 class and the Lewis − S 25-40 class, and the D > 1.063 fraction corresponds to the Lewis − S 1-10 class and Cohn fraction IV + V + VI.

It is evident that there is good agreement with the data published by Gofman, Glazier, Tamplin, Strisower, and deLalla (17) except for the D 1.019–1.063 fraction in women. This may be due to the small size of our sample. Recently, preliminary results of high density lipoprotein analysis have been published by the Donner Laboratory group (18) and these also appear to give values similar to our D > 1.063 fraction. The data of Lewis and Page (19) differ greatly from our data and from the data of others. In our hands their method of preparative ultracentrifugation fails to float all the cholesterol. This would account in part for their lower results. Our data agree well with the data of Russ, Eder, and Barr (3) obtained by Cohn fractionation. Not shown in the table is the comparison of our D < 1.063 fraction with their fraction I + III. Our mean value for total cholesterol in this fraction is 120 mg. per 100 ml. as compared to their value of 125. The cholesterol-phospholipid ratios obtained by these two methods also agree closely. Data obtained by zone electrophoresis (4–6) also agree closely with our data.

The observations on the patients with diseases predisposing to atherosclerosis confirm and extend those of McGinley, Jones, and Gofman (20) who found that hyperlipoproteinemia may involve the D < 1.063 flotation fractions to a variable degree. While the number of patients is small it is of interest that the cholesterol in the D > 1.063 fraction is reduced in only one of the patients having diseases predisposing to atherosclerosis as contrasted to the finding of Barr, Russ, and Eder (21) that cholesterol in Cohn fraction IV + V + VI was usually markedly reduced. This apparent discrepancy requires further investigation.

The results obtained in patients with liver disease corroborate previous observations (4, 5, 9) regarding the marked lowering of the alpha lipoproteins in obstructive jaundice. Eder, Russ, Pritchett, Wilber, and Barr (9) also found alterations in the chemical composition of the low density lipoproteins. Our data further show that this alteration involves both subfractions of this group. Since "pure" lipoproteins were not isolated by this procedure, it is apparent that these alterations in composition may possibly reflect changes in the proportions of different lipoproteins contained in the fractions. The altered chemical composition of the D 1.019–1.063 fraction could well account for its increased mean flotation rate in the analytical ultracentrifuge (22) and its poor resolution at density 1.019 from the lower density lipoproteins in the preparative ultracentrifuge.

The data further illustrate the complex nature of the cholesterol-phospholipid ratio of whole serum. Since in normocholesterolemic individuals the ratio in the D < 1.019 fraction is similar to that of serum, alterations in the concentration of this component will not appreciably alter the whole serum ratio. On the other hand, since the ratios in the D 1.019–1.063 and D > 1.063 fractions are appreciably different from the serum value, changes in the proportions of these components could alter the serum ratio significantly. Equally important, however, in determining the cholesterol-phospholipid ratio in serum are the

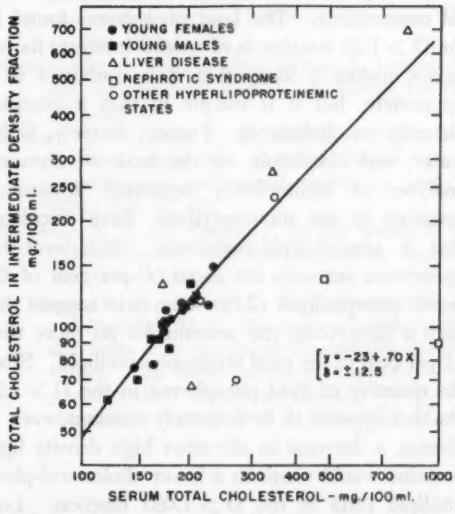

FIG. 1. RELATION OF TOTAL CHOLESTEROL IN D 1.019–1.063 FRACTION TO SERUM TOTAL CHOLESTEROL

Regression line was calculated from data on 10 young females and 10 young males by method of least squares. δ = standard deviation from regression.

alterations in lipoprotein composition of the fractions that occur with disease.

In Figure 1 is shown the correlation between the serum total cholesterol and the total cholesterol in the D 1.019–1.063 fraction in young adults and in the patients with hyperlipoproteinemia. As could be predicted *a priori* this correlation is good in young adults, since the D 1.019–1.063 fraction is the major cholesterol-containing component. The figure also shows that it is not possible to apply this correlation to the prediction of D 1.019–1.063 lipoprotein concentration from the serum total cholesterol in hyperlipoproteinemic states. In only two of the nine hyperlipoproteinemic subjects does the cholesterol in the D 1.019–1.063 fraction fall within one standard deviation of the regression line calculated for young adults. The correlation between the total cholesterol in the other fractions and the serum total cholesterol is poor. Likewise, intercorrelations between the total cholesterol contents of the various fractions are poor.

Study of the lipoproteins of D 1.063–1.21 shows, in agreement with observations of deLalla and Gofman (7), that these consist of more than one density class. The protein analysis shows that the different flotation classes also differ in chemical composition. The lipid phosphorus found in the D > 1.21 fraction is of special interest; its behavior makes it likely that it is contained in a lipoprotein, but it is unique in that it contains virtually no cholesterol. Turner, Snavely, Goldwater, and Randolph, on the basis of chemical analyses of incompletely separated lipoprotein fractions in the ultracentrifuge, have calculated that a phospholipid-containing, cholesterol-free lipoprotein accounts for about 60 per cent of the serum phospholipid (23). Our data suggest that such a lipoprotein can account for no more than 15 per cent of the total serum phospholipid. Since the quantity of lipid phosphorus in the D > 1.21 fraction appears to be relatively constant, even in disease, a decrease in the other high density lipoproteins would result in a lower cholesterol-phospholipid ratio in the D > 1.063 fraction. Low cholesterol-phospholipid ratios in the D > 1.063 fraction are seen in the five hyperlipoproteinemic individuals with low concentrations of cholesterol in this fraction (Table III).

SUMMARY

1. A method for the analysis of serum lipoproteins is described. Lipoprotein fractions are isolated by repeated ultracentrifugations after progressively raising the solvent density. The separated fractions are characterized by lipid and protein analysis. It is possible to separate several lipoprotein fractions from the same serum sample. Routinely three fractions of densities < 1.019, 1.019–1.063, and > 1.063 have been analyzed. The accuracy of the procedure is limited by the accuracy of the chemical methods employed.

2. Data obtained in young adults demonstrate that approximately two-thirds of the serum cholesterol and half the lipid phosphorus are contained in the density 1.019–1.063 fraction. The quantity of lipid is greater in the density < 1.019 fraction and less in the density > 1.063 fraction in young men as compared with young women. The ratio of total cholesterol to phospholipid is similar to that of serum in the density < 1.019 fraction, considerably greater in the density 1.019–1.063 fraction, and considerably less in the density > 1.063 fraction.

3. In disease hyperlipoproteinemia involves one or both of the two lower density fractions. The density > 1.063 fraction is variably affected; in biliary obstruction it is markedly reduced. Alterations in composition of the lipoprotein fractions also occur; these are most striking in biliary obstruction.

4. In animal sera wide variations in the distribution and composition of lipoproteins occur. A greater proportion of the lipid is associated with the density > 1.063 fraction than in man.

5. Subfractionation of the density > 1.063 fraction demonstrates two fractions containing cholesterol and lipid phosphorus of densities 1.063–1.125 and 1.125–1.21. The fraction of density > 1.21 contains 10 to 15 per cent of the serum lipid phosphorus but practically no cholesterol. This phosphorus is non-dialyzable and migrates with the alpha$_1$-albumin fraction in starch electrophoresis (see addendum).

Addendum

Since this paper was submitted for publication it has been reported by Hack that about 10 per cent of the phosphorus extracted from human serum with ethanol-ether is insoluble in petroleum ether and can be ac-

counted for by a peptide containing serine phosphate, threonine, and glutamic acid (Federation Proc., 1955, **14**, 222). We have found that the zinc hydroxide precipitable, ethanol-acetone soluble phosphorus in 1.21 density infranates is completely water soluble and insoluble in petroleum ether. The amino acids serine, threonine, glutamic acid, glycine, alanine, and leucine or isoleucine have been found in acid hydrolysates of the water soluble fraction. Further work on the nature of the phosphate in this fraction is in progress.

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TISSUE DISTRIBUTION OF I^{131} LABELED HUMAN SERUM ALBUMIN FOLLOWING INTRAVENOUS ADMINISTRATION

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Previous studies utilizing I^{131} labeled human serum albumin have revealed that approximately 60 per cent of the total exchangeable albumin of nonedematous subjects is in extravascular locations (1, 2). Following intravenous administration, approximately 4 to 7 days are required for essentially complete equilibration of the labeled albumin between plasma and extravascular tissues (2, 3). In almost all cases the plasma disappearance curves, appropriately corrected for metabolic degradation, manifest two major components with half times of approximately 3 to 5 hours and 24 to 30 hours, respectively, suggesting that there are at least two groups of albumin containing extravascular compartments which equilibrate with plasma at different rates (2, 3). However, the distribution of extravascular albumin and the anatomical sites of the rapidly and slowly equilibrating extravascular compartments have not yet been quantitatively defined although previous studies utilizing massive doses of heterologous serum proteins in rabbits established the presence of these proteins in the extravascular phase of muscle 48 hours after intravenous administration (4).

During the course of recent investigations on the fate of I^{131} labeled human serum albumin in heart failure (3), the deaths of two subjects, 1 and 22 days, respectively, following the intravenous administration of the labeled albumin, offered the opportunity for post mortem analysis of radioactivity in the various tissues. The times of death were such that comparison between the two cases yielded suggestive information regarding the distribution of iodoalbumin and the sites of the slowly and rapidly equilibrating extravascular compartments. Further observations were then made on skin and muscle tissue removed during surgical operations.

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METHODS

The subjects were hospitalized male patients on the Medical and Surgical Services of the Veterans Administration Hospital, Bronx, N. Y.

Patient V. P. had arteriosclerotic heart disease and congestive heart failure and died suddenly with a myocardial infarction 24 hours after the intravenous administration of 112 μc of I^{131} labeled human serum albumin. Patient A. M. had rheumatic heart disease, aortic insufficiency, congestive heart failure and persistent proteinuria and expired in pulmonary edema 22 days after receiving 107 μc of labeled albumin. In the interval between administration of the tracer protein and the deaths of the patients, serial blood samples were taken and complete urinary collections were made. Ten drops of Lugol's solution were administered daily to inhibit the uptake of free iodide by the thyroid. The plasma concentration curves of I^{131} tagged albumin which were obtained during life were extrapolated to the time of death in each patient. In each case post mortem examination was performed 24 hours after death at which time the tissues were obtained for analysis.

Five specimens of muscle and three specimens of skin were obtained during surgical procedures from five patients fifteen to twenty minutes after the intravenous administration of I^{131} labeled albumin. In addition, ten muscle specimens and eight skin specimens were obtained from twelve patients one to eleven days after administration of I^{131} labeled albumin. Plasma samples were obtained simultaneously with all surgical tissue specimens which were taken primarily from the thoracic and abdominal walls.

Representative samples of all tissues were trimmed of large blood vessels, patted free of excess blood, placed into Kimbal screw top culture tubes, and weighed. Care was taken to strip all subcutaneous tissue from skin samples. The tissues were then assayed for radioactivity in a well-type scintillation counter with a sensitivity of 1.00×10^6 cts. per min. per μc I^{131}.

The small vessel plasma contents of muscle and skin were determined from the total radioactivities of these tissues relative to those of plasma 15 to 20 minutes after the intravenous administration of I^{131} labeled albumin. It was assumed that negligible quantities of the labeled protein had passed into the extravascular spaces at this time. The small vessel plasma contents of other tissues were taken from the values given by Gibson, Peacock, Seligman, and Sack (5). The albumin-I^{131} contents of

"bloodless" tissues at later times were then estimated by applying these corrections for plasma content to the observed total tissue radioactivities.

Serum albumin concentrations were determined according to the method of Kingsley (6).

RESULTS

A—Post mortem tissue examinations in subjects V. P. and A. M.

Patient V. P. expired only 24 hours following the administration of the tracer protein and therefore distribution was presumed to be incomplete in the slowly equilibrating extravascular compartments. At the time of death 40 per cent of the iodoalbumin had left the blood stream. In patient A. M., distribution was complete at the time of death (22 days following administration of albumin-I^{131}), and specific activities of pleural fluid and plasma were found to be identical (0.00150 per cent of administered albumin-I^{131} per gram of albumin). Sixty per cent of the total exchangeable albumin was located extravascularly.

The results of the radioactive tissue assays are presented in Table I. With the exception of dura mater in subject A. M., no organ in either patient had a higher concentration of radioactivity than the plasma. The thyroid and gastric glands in subject A. M. might have shown a higher concentration of radioactivity if the administration of Lugol's solution had not reduced their capacity to concentrate radioiodide released from the degraded protein. The extremely high relative activity of the dura mater in subject A. M. was probably due to contamination from an external source.

The strikingly low concentration of iodoalbumin in the livers of both patients is of particular interest. The extravascular exchangeable albumin content of the entire liver was equivalent to the albumin contained in 77 ml. of plasma and 39 ml. of plasma in patients V. P. and A. M., respectively. Including blood content, exchangeable albumin

TABLE I

Tissue distribution of I^{131} labeled albumin

	Subject V. P.*				Subject A. M.†			
		Tissue *cts./min./gm.* / Plasma *cts./min./ml.*		Albumin content of entire bloodless organ in volume equivalents of plasma (*ml.*)		Tissue *cts./min./gm.* / Plasma *cts./min./ml.*		Albumin content of entire bloodless organ in volume equivalents of plasma (*ml.*)
Tissue	Organ wt. in *gm.*	Whole tissue	Bloodless tissue‡		Organ wt. in *gm.*	Whole tissue	Bloodless tissue‡	
Aorta		8.8×10^{-2}	$\times10^{-2}$			30.9×10^{-2}	$\times10^{-2}$	
Brain	1,440	1.6	0.5	7				
Dura		14.4				187.0		
Fat		8.4				15.6		
Ileum		20.5	15.4			13.9	8.8	
Stomach		14.7	10.7			63.0	58.0	
Kidney	130, 160	11.6	0.0	0	200, 210	16.2	3.5	12
Liver	1,690	15.0	5.7	77	1,430	14.8	3.3	38
Lymph nodes								
Tracheal		10.3				22.1		
Mesenteric		16.1						
Lungs	610, 1080	14.6	3.7	50	630, 700	27.2	16.5	171
Muscle								
Cardiac	630				760			
noninfarcted		11.4	7.5	44		16.9	11.8	80
infarcted		18.3						
Skeletal								
psoas		4.4	3.3			10.5	7.9	
Pancreas	175	12.1			130	21.0		
Spleen	340	20.7	14.6	29	300	19.2	12.4	22
Testes		13.1						
Thyroid						91.0		
Bile						16.6		
Pleural Fl.						45.8		
Plasma		100.0				100.0		

* Pt. V. P. expired 24 hours following intravenous administration of I^{131} labeled human serum albumin.
† Pt. A. M. expired 22 days following intravenous administration of I^{131} labeled human serum albumin.
‡ Whole tissue counts corrected for plasma content of tissue as described under Methods.

TABLE II

Small vessel plasma volumes of skin and muscle (per cent by weight of whole tissue) *

Subject	Skin	Muscle
N. D.	1.2	2.7
V. C.	2.8	2.9
H. M.	1.0	2.2
J. A.		2.9
A. F.		2.7
Mean	1.9	2.7

* Based on relative tissue/plasma radioactivities 15 to 20 minutes after intravenous administration of I^{131} labeled albumin.

present in the livers of these subjects was less than 10 grams or at most, 3 to 4 per cent of the total exchangeable albumin in the body.

Liver, spleen, and small intestine failed to show any increase in concentration of iodoalbumin relative to that of plasma between 1 and 22 days. The other viscera showed varying increments, but the total iodoalbumin content of these organs was only a small fraction of the total exchangeable albumin, even when the blood content is included.

Both skeletal muscle and noninfarcted cardiac muscle revealed a definite increase in "bloodless" tissue radioactivity between the 1st and 22nd days. However, since both patients were in heart failure at the time of death, it was not possible to establish values for edema free muscle other than to note that the maximum value was not greater than about 10 per cent that of plasma. The higher concentration of radioactivity in the infarcted cardiac muscle is explained by the seepage of blood into the infarcted area.

B—Muscle and skin samples obtained at operation

The mean plasma volumes of muscle and skin as determined from tissues obtained 15 to 20 minutes after I^{131} albumin administration were 2.7 per cent and 1.9 per cent, by weight, respectively (Table II). Eighteen to 24 hours later "bloodless" muscle had a concentration of only 0.6 per cent that of the plasma but this value rose gradually over the next four to five days to 3.5 per cent after which no further increase occurred (Figure 1). "Bloodless" skin, however, showed a more rapid initial increase, reaching a concentration of 11.0 per cent relative to the plasma at eighteen hours and this was followed by a further gradual [illegible] to 25 to 30 per cent by the end of 5 to 7 days (Figure 1).

DISCUSSION

Since the specific activity of labeled albumin in accessible extravascular compartments such as ascitic fluid (7) and pleural fluid (present studies) eventually becomes virtually equal to that in plasma, it seems reasonable to conclude that albumin-I^{131} is distributed elsewhere in the same manner as endogenous serum albumin, whatever reservations may yet remain (2, 3) regarding the relative rates of metabolism of endogenous albumin and albumin-I^{131}. Therefore, in the discussion below, it is assumed that the distribution of albumin-I^{131} reflects that of endogenous serum albumin.

Albumin in viscera

Since radioiodide is excreted rapidly following its liberation from degraded protein (2), the concentration of radioactivity in tissues, under the conditions of these studies, may be regarded as due almost entirely to protein. In all tissues except thyroid and stomach [3] (which selectively

[3] Iodine concentrations in these organs was partially inhibited by the administration of Lugol's solution.

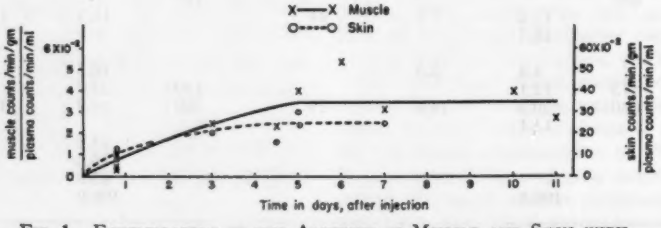

FIG. 1. EQUILIBRATION OF THE ALBUMIN OF MUSCLE AND SKIN WITH PLASMA ALBUMIN

Tissue counts have been corrected for plasma content.

concentrate iodine) nonprotein-bound radioiodine contributes an insignificant contamination as in plasma where 98 per cent or more of the radioactivity can be shown to be albumin-I^{131} (2). However, pathologic conditions were probably responsible for higher than normal concentrations of albumin-I^{131} in some tissues of the subjects studied here at autopsy. The concentration of radioactivity in the lungs of patient A. M. was probably elevated by the presence of vascular congestion and pulmonary edema whereas the correction for plasma content was based on normal lung tissue. Also, patient A. M. had considerable proteinuria (3 to 5 gm. per day) and at least some of the albumin-I^{131} in his "bloodless" kidney tissue may have been due to protein in the renal collecting system rather than in the interstitium. Therefore, the total exchangeable albumin content of normal viscera may be even lower than that observed here. In subject A. M. the total extravascular albumin content of heart, liver, spleen, kidneys, and lungs amounted to about one-tenth of the circulating plasma content.

In view of the low hydrostatic pressure existing in the portal venous system relative to the plasma oncotic pressure, it has been suggested on theoretical grounds that the extracellular fluids of the liver must maintain a high protein concentration in order to resist desiccation (8). Although *in vivo* studies in the human subject (2), in the dog (5) and in the rabbit (9), have failed to demonstrate significant hepatic extraction of I^{131} labeled albumin from the first circulation (9) to the first hour (2, 5) following its administration, the possibility of hepatic concentration at later intervals had not been investigated. The observations presented here indicate that even after 22 days there is no appreciable quantity of exchangeable albumin in the extravascular spaces of the liver and hence the volume of the extracellular space of the liver must be small. This is confirmed by the lack of histologic evidence for a significant interstitial space in hepatic parenchyma.

Albumin in skin and muscle

The concentration of exchangeable albumin in whole skin exclusive of its blood vessel content is about 25 to 30 per cent that in plasma. If the serum albumin of skin is located solely in the interstitial fluid, estimated at 60 per cent of skin weight (10), then the concentration in this fluid is about 45 per cent that in plasma or normally about 2.0 grams per 100 ml. Estimates of total skin mass vary greatly but the most reasonable values appear to be about 6 to 7 per cent of body weight (11, 12). Since the plasma volume is about 4 per cent of body weight (13) and contains about 40 per cent of the total exchangeable albumin in the body, the total albumin content of skin accounts for about 18 per cent of the total exchangeable albumin. The high albumin concentration in whole skin is undoubtedly related to the large volume of extracellular fluid in this tissue and may explain the relatively large quantity of extravascular albumin observed in a myxedematous subject reported elsewhere (3).

The concentration of exchangeable albumin in extravascular compartments of muscle is only 3.5 per cent that in plasma, but since muscle constitutes 40 to 45 per cent of body weight (11, 14), this tissue accounts for about 15 per cent of the total exchangeable albumin. Assuming that all the albumin of muscle is located in the interstitial fluid estimated at 10 per cent (15) to 16 per cent (16) of muscle, its concentration in this fluid is then approximately 35 per cent to 21 per cent of the plasma concentration or normally about 1.5 grams to 0.92 gram per 100 ml., respectively.

Table III summarizes the data pertaining to the major sites of albumin distribution. Almost four-fifths of the total exchangeable albumin can be accounted for in plasma, skin, muscle and the thoracic and abdominal viscera listed. The remainder must include that present in other viscera, subcutaneous tissue, fat, lymphatic trunks, and bone marrow. Mixing of intravenously administered iodoalbumin with the extravascular albumin pools of muscle, certain viscera, and at least an ap-

TABLE III

Major sites of albumin distribution

	Per cent of total exchangeable albumin
Plasma	40
Skin	18
Muscle	15
Heart, lungs, liver, kidney, spleen	4
Total accounted for in above sites	77

preciable portion of skin, requires 4 to 5 days for complete equilibration. These observations are in agreement with the half times of disappearance following direct injection of albumin-I^{131} into skin and muscle (*2*). Other viscera, such as liver and spleen, and possibly some compartment of skin have albumin pools which appear to equilibrate with plasma albumin within a day or less. It must be emphasized that the half times of the 2 compartments of distribution observed in the plasma disappearance curves previously described (*2*) and discussed above are not the half times for equilibration of the 2 groups of extravascular compartments brought to light by the characteristics of the plasma concentration curves. The latter half times cannot be calculated from the plasma disappearance curves alone (*7*).

SUMMARY AND CONCLUSIONS

1. Tissue radioactivities were determined at autopsy in two patients who died 1 and 22 days following the intravenous administration of I^{131} labeled albumin and in surgical specimens of skin and muscle obtained from human subjects 15 minutes to 11 days following iodoalbumin administration.

2. Plasma and the extravascular spaces of skin, muscle and the large thoracic and abdominal viscera account for 75 to 80 per cent of the total exchangeable albumin. Skin and muscle contain one-half of the total extravascular albumin. The extravascular space of the liver contains less than 1 per cent of the body's total exchangeable albumin.

3. Four to 5 days are required for essentially complete equilibration of the extravascular exchangeable albumin pools of skin and muscle with plasma albumin.

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DISTRIBUTION AND METABOLISM OF I^{131} LABELED HUMAN SERUM ALBUMIN IN CONGESTIVE HEART FAILURE WITH AND WITHOUT PROTEINURIA

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The role of serum albumin in the causation of cardiac edema has been disputed. Starling (1) postulated that circulatory stasis resulting from cardiac insufficiency is responsible for anoxia and increased permeability of the capillary membrane, the consequence of which is the passage of albumin and water into the extravascular spaces. Although Smirk (2) observed evidence of increased capillary permeability to water and crystalloids in congestive heart failure, Stead and Warren (3) demonstrated, by direct analysis, that the protein concentration of cardiac edema fluid was not elevated above that of the edema fluid produced by tourniquet stasis in normal subjects. Other studies have directed attention to the low serum albumin concentrations occasionally encountered in heart failure (4–7), and have suggested or implied (4, 8) a causal relationship to the pathogenesis of cardiac edema. Abnormalities in liver function tests and morphologic evidence of liver injury in severe congestive failure (9–14) have appeared to strengthen the possibility that hepatic synthesis of albumin may be impaired in this condition. Alternatively, low serum albumin concentrations have been attributed to inadequate dietary protein intake or poor absorption from a congested gastrointestinal tract (15, 16).

Since the plasma concentration of albumin is not an adequate measure of the total quantity of circulating and extravascular albumin stores, it was of interest to measure the amount of total exchangeable albumin in subjects with heart failure employing I^{131} labeled albumin as a tracer. The rate of metabolism of I^{131} labeled albumin was also studied with the aim of evaluating the ability of the subject in heart failure to synthesize albumin. Although there may yet remain some reservations regarding the validity of this tracer in the investigation of serum albumin metabolism, the present study was designed to eliminate several objections posed previously (17). Observations on the effect of proteinuria on albumin distribution and metabolism are also presented.

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[2] Dazian Foundation Fellow.

METHODS

Twenty hospitalized patients were divided into groups according to the lot of I^{131} labeled albumin employed. There were ten patients in heart failure, five of whom had proteinuria, and ten non-cardiac control subjects, two of whom had marked proteinuria. In each of the first three groups there were cardiac and non-cardiac control subjects: Group IV consisted of two non-cardiac control subjects and one patient with myxedema. In addition, the data pertaining to ten subjects without heart failure or proteinuria, who were studied in a manner identical with that in the present study, using lots of I^{131} labeled albumin free from excessive amounts of rapidly degraded components (17), are included as controls. Both control groups included normal volunteers as well as patients with such chronic illnesses as rheumatoid arthritis, uncomplicated duodenal ulcer, and well controlled diabetes mellitus. Diet and therapy were not regulated in any particular fashion for the purpose of this study but were dictated by individual requirements. For the experimental group only patients with markedly advanced and intractable congestive heart failure were chosen.

I^{131} labeled albumin was prepared according to the method of Newerly (18) and contained 1 to 2 iodine atoms per molecule of protein. The method consists essentially in the oxidation of iodide to elemental iodine by the method of Pressman and Eisen (19), extraction of the oxidized iodine with chloroform, and slow addition of the chloroform-iodine solution to an appropriate dilution of pooled human serum albumin. Non-protein bound iodine was removed by dialysis against normal saline solution for 48 hours following which the solutions were sterilized by passage through Seitz filters and cultured before use. The final products contained less than 2 per cent non-protein bound radioactivity as determined by precipitation with 10 per cent cold trichloracetic acid.

Weighed solutions of the tagged albumin containing 85 to 115 μc I^{131} and 1 to 10 mg. protein were administered intravenously. Thyroidal uptake of radioiodine was

inhibited by the administration of 10 drops of Lugol's solution daily throughout the period of observation. Heparinized blood samples were taken without tourniquet stasis 15 minutes, 2, 6, 24, 30, and 48 hours after injection and at almost daily intervals thereafter for the next 25 to 35 days. Complete urinary collections were made daily. All samples were assayed in a well type scintillation counter with a sensitivity of 1.00×10^6 counts per min. per μc I^{131} above a background of about 200 counts per min. Serum albumin concentrations were determined at weekly intervals by the method of Kingsley (20). Urinary albumin concentrations were determined by the biuret method of Foster, Rick, and Wolfson (21).

The data were analyzed according to methods previously described (17), and only a brief summary is given here for the purpose of orientation. Plasma volume was determined from the I^{131} labeled albumin space of dilution, 15 minutes after administration (22). Total circulating albumin was calculated as the product of serum albumin concentration and plasma volume, and total exchangeable albumin (TEA) was calculated from the total plasma albumin and the relative apparent spaces of distribution at 15 minutes and at distribution equilibrium (Figure 1). When necessitated by incompleteness of urinary collections, appropriate corrections for the apparent spaces of distribution were made as previously described (17). Extravascular albumin was determined from the difference between total exchangeable albumin and total circulating albumin.

In the steady state, total albumin synthesis is equal to the sum of albumin degraded and albumin lost in the urine and was calculated independently from urine and plasma data as previously described (Figures 1, 2) (17):

1) Total albumin synthesis equals the product of total exchangeable albumin and the rate constant of decrease of I^{131} labeled albumin in plasma after distribution equilibrium (Figures 1, 2).

2) Total albumin synthesis equals the product of total exchangeable albumin and the fraction of total iodoalbumin remaining in the body which is excreted daily after distribution equilibrium (Figures 1, 2).

In the subjects with proteinuria, total urinary radioactivity was fractionated by precipitation with cold 10 per cent trichloracetic acid into that derived from albumin metabolism (non-protein bound urinary radioactivity) and that resulting from albuminuria (protein bound urinary radioactivity). The partition of radioactivity between the two fractions was strikingly constant from day to day in individual subjects (Figure 2).

Albumin loss due to proteinuria was calculated from the product of the total exchangeable albumin and the

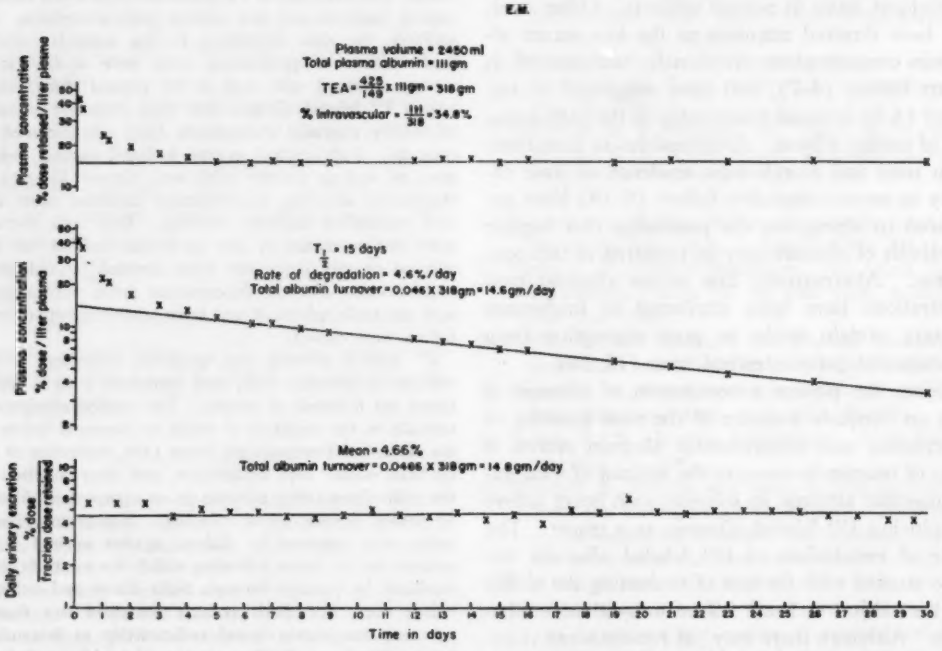
E.M.
Plasma volume = 2450 ml
Total plasma albumin = 111 gm
TEA = 425/149 x 111 gm = 318 gm
% Intravascular = 111/318 = 34.8%
Plasma concentration % dose retained/liter plasma
$T_{\frac{1}{2}}$ = 15 days
Rate of degradation = 4.6%/day
Total albumin turnover = 0.046 X 318 gm = 14.6 gm/day
Plasma concentration % dose/liter plasma
Mean = 4.66%
Total albumin turnover = 0.0466 X 318 gm = 14.8 gm/day
Daily urinary excretion % dose
Fraction dose retained
Time in days

FIG. 1. TYPICAL SET OF CURVES AND CALCULATIONS (A NON-CARDIAC SUBJECT WITHOUT PROTEINURIA)

The mean per cent urinary excretion $\left(\frac{\%\text{ dose}}{\text{fraction dose retained}}\right)$ was obtained from the values between the sixth and thirtieth day inclusive.

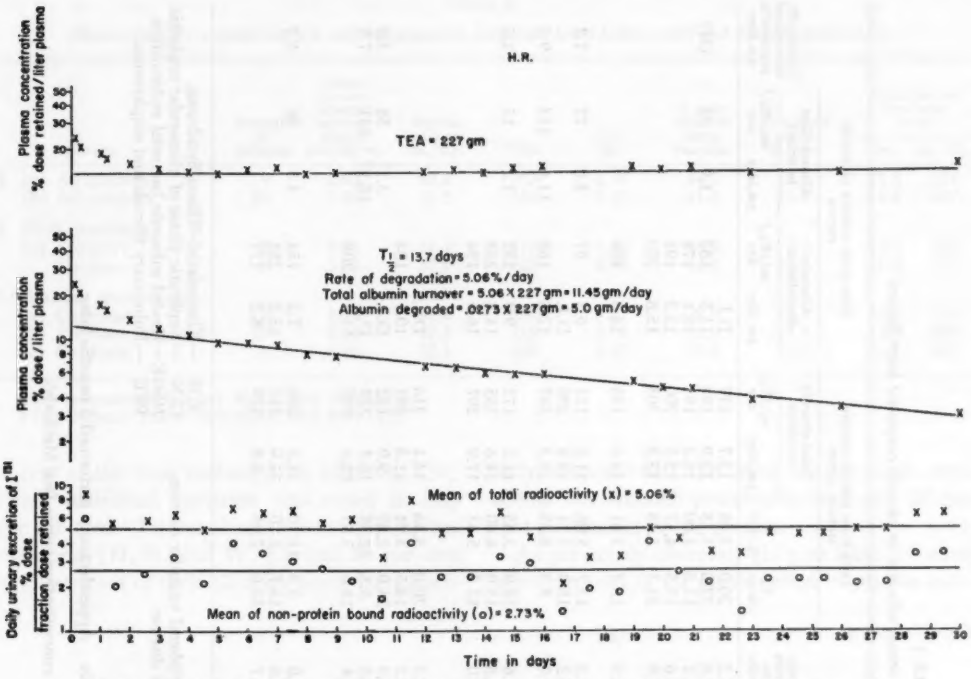
H.R.
Plasma concentration % dose retained/liter plasma
TEA = 227 gm
$T_{\frac{1}{2}}$ = 13.7 days
Rate of degradation = 5.06%/day
Total albumin turnover = 5.06 X 227 gm = 11.45 gm/day
Albumin degraded = .0273 X 227 gm = 5.0 gm/day
Plasma concentration % dose/liter plasma
Daily urinary excretion of I^{131} % dose / fraction dose retained
Mean of total radioactivity (x) = 5.06%
Mean of non-protein bound radioactivity (o) = 2.73%
Time in days

FIG. 2. TYPICAL SET OF CURVES AND CALCULATIONS (A CARDIAC SUBJECT WITH PROTEINURIA)

The mean per cent urinary excretions $\left(\frac{\%\text{ dose}}{\text{fraction dose retained}}\right)$ were obtained from the values between the sixth and thirtieth days inclusive.

fraction of total radioactivity remaining in the body excreted in protein bound form daily. The values obtained by this method agreed reasonably well with those obtained by direct chemical analysis of urinary albumin (Table I). In two subjects with proteinuria, A. M. and M. C., urinary radioactivity data were incomplete and radioactive albumin losses in the urine were calculated from the plasma I^{131} labeled albumin disappearance curves and the fraction of total urinary radioactivity which was precipitable.

RESULTS

Results for individual subjects are given in Table I, and the mean values for different groups are compared in Table II. Serum albumin concentrations varied considerably from patient to patient, but the mean concentration in the cardiac group as a whole was only slightly lower than that of the present non-cardiac group and insignificantly lower than that of the control group studied previously. Cardiac subjects without proteinuria demonstrated no decrease in serum albumin level.

Plasma volumes in the subjects with heart failure were definitely increased over those of both non-cardiac control series, which is in agreement with many previous studies (23–27). Total circulating albumin was therefore actually greater in cardiac subjects without proteinuria (165 gm.) than in control subjects (140 gm.). The mean value for total exchangeable albumin in the non-proteinuric cardiac group (5.45 gm. per Kg. body weight) was higher than in the two control groups (5.07 gm. per Kg. and 5.18 gm. per Kg., respectively). Two of the proteinuric cardiac subjects (H. R. and W. J.) had diminished values for circulating and total exchangeable albumin but the lowest values were obtained in the two patients with glomerular nephritis who were free of heart failure. These findings were not related to differences in the degree of proteinuria.

Average values for the intravascular-extravascular partition of albumin were quite similar in all groups, with the plasma containing about 40

TABLE I

Distribution and degradation of I^{131} labeled human serum albumin in chronic congestive heart failure *

Group	Subject	Diagnosis	Age	Weight Kg.	Mean serum albumin concentration gm./100 ml.	Plasma volume ml.	TEA gm.	TEA gm./Kg.	% Iodoalbumin intravascular	Data from plasma concentration curves: $T_{\frac{1}{2}}$ days	Data from plasma concentration curves: % /day	Data from plasma concentration curves: Total albumin synthesis gm./day	Data from plasma concentration curves: Total albumin synthesis mg./Kg./day	Data from urinary excretion curves: Albumin degradation gm./day	Data from urinary excretion curves: Albumin degradation mg./Kg./day	Data from urinary excretion curves: Albumin lost in urine† gm./day	Data from urinary excretion curves: Albumin lost in urine† mg./Kg./day	Chemically determined proteinuria gm./day
I	C. E.	UHD	47	88.7	4.24	4,000	454	5.13	37.2	20.0	3.46	15.7	177	D.I.				
	C. W.	ASHD	53	70.4	3.95	4,220	442	6.28	37.6	22.0	3.15	13.9	198	11.5	163	1.9	28	(3†)
	J. G.	RHD	61	84.9	4.90	3,870	406	4.78	46.7	17.3	4.00	16.3	191	15.1	178			
	R. H.	P, Control	25	78.5	4.51	3,080	352	4.58	39.6	15.5	4.47	15.7	200	15.2	194			
	E. F.	Conv. malaria, Control	24	76.9	5.18	3,490	490	6.38	36.9	21.5	3.23	15.8	205	15.6	203			
	D. G.	ASPVD, Control	57	75.0	4.90	2,565	383	5.12	32.9	18.2	3.81	14.6	195	14.5	194			
II	H. R.	HHD	58	66.0	3.10	4,260	227	3.44	58.5	13.7	5.06	11.5	174	6.5	97	5.0	77	7.2
	R. J.	ASHD	73	65.8	4.24	3,905	371	5.64	40.2	18.8	3.71	13.8	209	D.I.				
	W. J.	ASHD, Diab M., K.W.	57	76.0	3.20	2,690	315	4.15	29.0	8.3	8.35	26.3	347	12.8	168	11.6	153	9.5
	F. B.	RHD, HSBE	29	65.3	3.71	3,890	291	4.44	49.6	18.0	3.85	11.2	172	9.9	152	1.1	17	1.5
	E. M.	Diab M., Control	43	57.3	4.52	2,450	318	5.54	34.8	15.0	4.60	14.6	255	14.8	258			
	E. W.	Duo. ulcer, Control	43	85.9	4.26	2,900	324	3.77	38.0	12.5	5.53	17.9	207	16.6	194			
III	A. H.	ASHD	58	65.5	3.71	3,860	408	6.20	35.3	20.0	3.46	14.1	216	D.I.				
	E. T.	RHD	65	54.6	3.73	3,080	302	5.53	38.2	18.5	3.74	11.3	207	10.1	184			
	A. M.	RHD	50	54.6	3.65	3,830	343	6.30	40.9	24.0	2.89	9.9	182	D.I.		3.1‖	56	3.0
	A. R.	G-N, Control	37	70.4	1.75	3,580	128	1.82	50.5	5.5	12.55	16.1	229	D.I.		10.1‖	141	7.0
	D. S.	P, Control	41	63.3	4.68	3,250	331	5.20	46.4	18.0	3.85	12.4	197	13.2	208			
IV	M. C.	G-N, PFE	56	46.8	1.76	2,400	98	2.10	43.0	5.9	11.70	11.5	246	7.2	154	4.5	96	4.2
	A. Z.‡	Appendicitis	25	60.5	4.86	2,665	291	4.81	44.6	13.7	5.10	14.9	247	15.2	251			
	M. Co.§	Myxedema, ASHD	64	68.0	3.73	3,120	427	6.43	26.7	33.6	2.06	8.8	129	8.2	121			

* TEA —Total exchangeable albumin.
D.I. —Data insufficient.
UHD —Undiagnosed heart disease.
ASHD—Arteriosclerotic heart disease.
RHD —Rheumatic heart disease.
P —Psychoneurosis.
ASPVD —Arteriosclerotic peripheral vascular disease.
HHD —Hypertensive heart disease.
Diab M.—Diabetes mellitus.
K.W. —Kimmelstiel-Wilson Syndrome.
G-N —Nephrotic phase of glomerular nephritis.
HSBE—Healed subacute bacterial endocarditis.
PFE —Pulmonary fibrosis and emphysema.

† Calculated from protein bound radioactivity in the urine.
‡ Study carried out during convalescence from an attack of acute appendicitis. Appendectomy performed 5 months later.
§ No definite evidence of congestive heart failure.
‖ Determined from precipitable fraction of urinary radioactivity and plasma concentration curve (see Methods).

TABLE II

Mean values for control subjects and subjects with congestive heart failure with and without proteinuria

	Groups	Number of subjects	Mean serum albumin concentration *gm./100 ml.*	Plasma volume *ml./Kg.*	TEA	TEA / Kg.	% Iodoalbumin intravascular	$T_{\frac{1}{2}}$ Plasma curve in days	Total albumin synthesis daily *gm.*	Total albumin synthesis daily *mg./Kg.*
I	(a) All controls*	9	4.05	43.8	302	4.37	40.7	14.0	14.8	220
	(b) All cardiacs	10	3.84	55.5	356	5.19	41.3	18.1	14.4	207
II	With proteinuria									
	(a) Controls	2	1.76	51.1	113	1.96	46.8	5.7	13.8	238
	Cardiacs	5	3.52	57.9	323	4.92	43.1	17.2	14.6	214
III	Without proteinuria									
	(a) Controls*	7	4.71	41.4	356	5.07	39.0	16.3	15.1	215
	(b) Previous controls†	10	4.18	47.5	325	5.18	38.9	17.8	13.0	205
	(c) Cardiacs	5	4.16	53.1	389	5.45	39.5	19.0	14.2	200

* Myxedematous subject M. Co. not included.
† See Berson, Yalow, Schreiber, and Post (17).

per cent of the total exchangeable albumin. Significant individual variation was noted in only three subjects. Two cardiac subjects with marked proteinuria (H. R. and W. J.) had 59 per cent and 29 per cent of their albumin stores intravascularly, respectively; the third subject with myxedema but without proteinuria had only 27 per cent circulating in the plasma.

As previously observed (17), at least two rates were involved in the equilibration of plasma iodo-

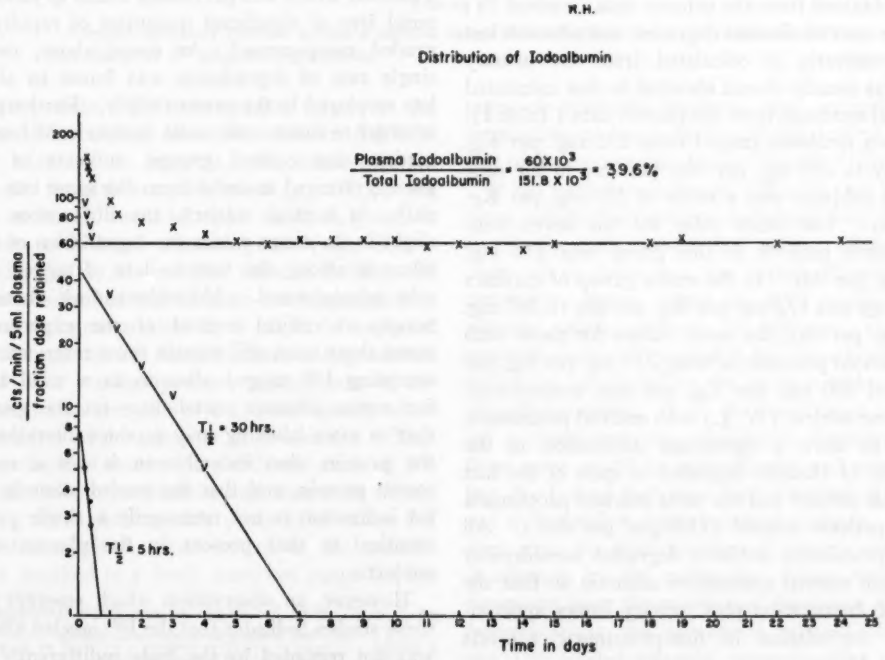
W.H.
Distribution of Iodoalbumin
Plasma Iodoalbumin / Total Iodoalbumin = 60 X 10³ / 151.8 X 10³ = 39.6%
cts/min/5ml plasma / fraction dose retained
T½ = 30 hrs.
T½ = 5 hrs.
Time in days

FIG. 3. ANALYSIS OF THE DISTRIBUTION CURVE OF IODOALBUMIN FOR PATIENT R. H., SHOWING 2 COMPONENTS WITH HALF-TIMES OF 5 HOURS AND 30 HOURS, RESPECTIVELY, INDICATING AT LEAST TWO SETS OF EXTRAVASCULAR COMPARTMENTS EQUILIBRATING WITH PLASMA IODOALBUMIN

albumin with extravascular albumin (Figure 3). The mean half times for decrease in the plasma concentration after distribution equilibrium in non-proteinuric subjects were shorter in the control groups ($T_{1/2} = 16.3$ and 17.8 days) than in the cardiac patients ($T_{1/2} = 19.0$ days). However, total exchangeable albumin was somewhat greater in the latter subjects so that the amount of albumin synthesized was approximately the same (Table II). In all cases a single rate of degradation was obtained after distribution equilibrium occurred (Figures 1, 2). In previous studies I^{131} labeled albumin prepared in this manner was also consistent in showing a single rate of degradation (17).

Since serum albumin levels remained essentially constant in all subjects throughout the study, steady state conditions were obtained and the rates of albumin synthesis were calculated according to each of the methods described. The values for synthesis given in Table I were determined from the plasma disappearance curves (method 1) but were generally in excellent agreement with those obtained from the urinary data (method 2); *i.e.*, the sum of albumin degraded and albumin lost via proteinuria as calculated from the urinary data was usually almost identical to that calculated for total synthesis from the plasma data (Table I). Albumin synthesis ranged from 195 mg. per Kg. per day to 255 mg. per Kg. per day in the nine control subjects with a mean of 220 mg. per Kg. per day. The mean value for the seven non-proteinuric patients in this group was 215 mg. per Kg. per day. In the entire group of cardiacs the range was 172 mg. per Kg. per day to 347 mg. per Kg. per day, the mean values for those with and without proteinuria being 214 mg. per Kg. per day and 200 mg. per Kg. per day, respectively. Only one subject (W. J.) with marked proteinuria failed to show a significant diminution in the quantity of albumin degraded in spite of the fact that this subject had the most marked proteinuria of all patients studied (11.6 gm. per day.). All other proteinuric subjects degraded considerably less than normal amounts of albumin so that the sum of degradation plus urinary losses approximated degradation in non-proteinuric subjects (Table I).

The patient with myxedema (M. Co.), who had arteriosclerotic heart disease but no definite evidence of heart failure, had the lowest rate of albumin synthesis (129 mg. per Kg. per day) of all subjects studied, although his total exchangeable albumin was the highest in terms of body weight (6.43 gm. per Kg.).

DISCUSSION

While the use of I^{131} labeled albumin for the investigation of the distribution of endogenous serum albumin is validated by the finding of essentially identical specific activities at distribution equilibrium in several measurable compartments such as plasma, pleural fluid (28), and ascitic fluid (17, 29), caution has been advised in its application to the study of endogenous albumin metabolism (17). Difficulties experienced previously (17) were related to differences in the rates of degradation of lots of iodoalbumin prepared by different methods and the presence of rapidly degraded components in many of the lots. In the present study these difficulties have been circumvented by preparing all batches of iodoalbumin by a method which was previously found to yield material free of significant quantities of rapidly degraded components.[3] As noted above, only a single rate of degradation was found in all the lots employed in the present study. Furthermore, in order to make more valid comparisons between cardiac and control groups, subjects of both groups received material from the same lots. Finally, in normal subjects the differences were slight in the mean values for degradation of iodoalbumin among the various lots of tagged albumin administered. Notwithstanding these attempts at critical control of the experimental cases, there must still remain some reservations in accepting I^{131} tagged albumin as a valid tracer for serum albumin metabolism on the grounds that *in vitro* labelling may produce alterations in the protein, that iodoalbumin is not a normal serum protein, and that the pooled albumin used for iodination is not necessarily a single protein identical to that present in the plasma of all subjects.

However, an observation which emerges from these studies indicates that the I^{131} labeled albumin was not regarded by the body indifferently as a

[3] Obvious alteration of the protein leads to rapid degradation (17, 30).

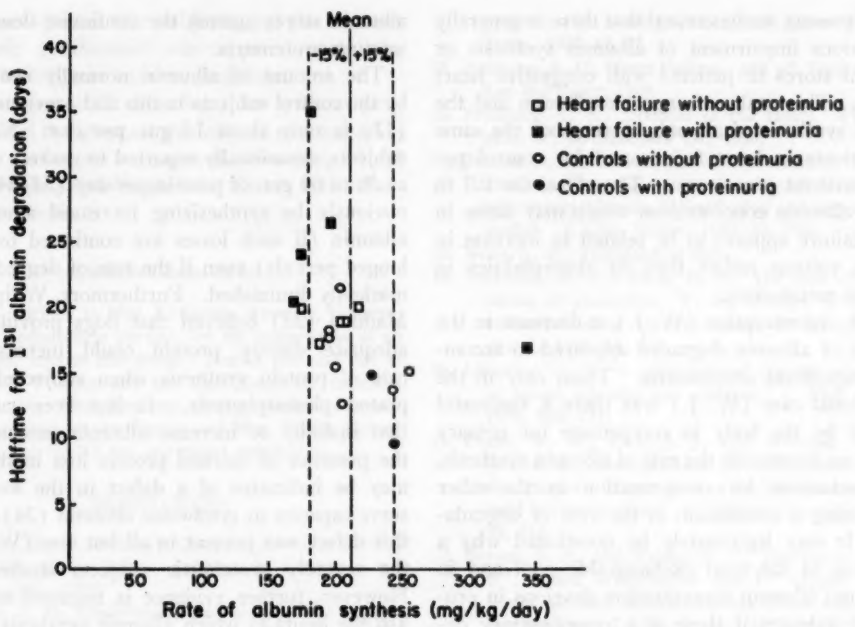

FIGURE 4

Albumin synthesis remains within a narrow range although there are wide variations in the half times for I^{131} albumin degradation.

foreign protein. This observation relates to the widely different half times for albumin degradation[4] obtained in subjects with small albumin pools due to persistent proteinuria (*e.g.*, $T_{1/2} = 9.4$ days in M. C. and 14.7 days in A. R.) and in subjects with large albumin pools (*e.g.*, $T_{1/2} =$ 35.0 days in A. M. and 26.7 days in C. W.). If the metabolism of iodoalbumin were unrelated to that of endogenous albumin it might be expected that the rate of I^{131} labeled albumin degradation would be a random process, independent of abnormalities in the size of the exchangeable albumin pool or of the presence of proteinuria. That the products of such widely different rates of I^{131} labeled albumin metabolism and almost as widely varying total exchangeable pools gave values for degradation which, when added to the urinary losses, resulted in a much narrower range for the rates of synthesis suggests very strongly that metabolism of the iodoalbumin did indeed reflect that of the endogenous serum albumin (Figure 4).

It has been demonstrated (31, 32) that the sodium sulfate fractionation technique may lead to spuriously high values for serum albumin in cases where there is a low serum albumin concentration; hence, values for total exchangeable albumin, total synthesis of albumin, and the amounts of albumin degraded may have been lower than those calculated for the patients with marked hypoalbuminemia. If so, the conclusions regarding a diminished rate of albumin degradation in these cases would be strengthened and even subject W. J. may then have had a normal synthesis and a diminished degradation of albumin. However, since the determination of urinary excretion of albumin by the radioactive iodine method is dependent upon the accuracy of the serum albumin determinations, the agreement between chemical and I^{131} labeled albumin determinations of urinary protein indicates that the serum albumin values were not grossly in error.

[4] These half times can be calculated from Table I, as follows: $\lambda = \frac{\text{grams of albumin degraded per day}}{\text{total exchangeable albumin}}$, where λ is the fraction of the total exchangeable albumin pool degraded per day. Then, $T_{\frac{1}{2}} = \frac{.693}{\lambda}$.

The present studies reveal that there is generally no obvious impairment of albumin synthesis or albumin stores in patients with congestive heart failure. The total exchangeable albumin and the rate of synthesis of albumin were about the same for patients in heart failure and for control patients without proteinuria. Therefore, the fall in serum albumin concentration which may occur in heart failure appears to be related to increase in plasma volume rather than to abnormalities in albumin metabolism.

With one exception (W. J.), a decrease in the amount of albumin degraded appeared to accompany significant albuminuria. Thus, only in the exceptional case (W. J.) was there a successful attempt by the body to compensate for urinary loss by an increase in the rate of albumin synthesis, the mechanism for compensation in the other cases being a diminution in the rate of degradation. It may legitimately be questioned why a reduction in the total exchangeable pool and in the serum albumin concentration develops in proteinuric subjects if there is a compensatory decrease in the rate of albumin degradation and a maintenance of normal levels of albumin production. However, it is common experience that many patients can tolerate a mild degree of proteinuria for prolonged periods without suffering a depletion of their protein stores, while in those with marked chronic proteinuria the limit to which degradation can be inhibited may be insufficient to prevent total albumin removal (degradation plus proteinuria) from exceeding replenishment by synthesis. Thus, protein losses in the patients studied here may well have been more prominent earlier in the course of disease (when serum albumin concentrations were as yet undiminished) and as little as a 10 per cent discrepancy between synthesis and total removal of albumin would have resulted in a 30 per cent deficit in total exchangeable albumin within a couple of months. Furthermore, it is probable that a decrease in albumin degradation is not effected until there has been some reduction in the serum albumin concentration and in the total albumin pool, since such changes would appear to be the most likely stimuli for this compensatory mechanism which acts to conserve the body's albumin stores. At this point the limited capacity of the liver to synthesize albumin may barely maintain the lowered albumin stores against the continued demands of wasting proteinuria.

The amount of albumin normally synthesized by the control subjects in this and previous studies (17) is only about 15 gm. per day. Nephrotic subjects, occasionally reported to excrete as much as 36 to 60 gm. of protein per day (33, 34), must obviously be synthesizing increased amounts of albumin (if such losses are continued over prolonged periods) even if the rate of degradation is markedly diminished. Furthermore, Whipple and Madden (35) believed that dogs provided with adequate dietary protein could increase their rate of protein synthesis when subjected to repeated plasmapheresis. It has been suggested that inability to increase albumin production in the presence of marked protein loss in the urine may be indicative of a defect in the liver's reserve capacity to synthesize albumin (34). If so, this defect was present in all but one (W. J.) of the severely proteinuric subjects studied here. However, further evidence is required to establish the limits to which albumin synthesis can increase in human subjects before it can be concluded that a normal rate of synthesis in the presence of marked proteinuria is evidence of a defect in ability to synthesize albumin. The present report provides insufficient data from which to derive conclusions on this point. However, Gutman (36) has suggested the possibility that a normal rate of synthesis may represent the maximal synthetic capacity in view of the fact that there is no clinical condition (other than dehydration) associated with an abnormally high serum albumin concentration.

SUMMARY AND CONCLUSIONS

1. The distribution and metabolism of I^{131} labeled human serum albumin were studied in 10 cardiac subjects with congestive heart failure and in 10 non-cardiac control subjects. Significant proteinuria was present in 5 of the cardiac and 2 of the non-cardiac patients.

2. Total exchangeable albumin, the intravascular-extravascular partition of exchangeable albumin, and the rate of albumin synthesis were not significantly altered in patients with congestive heart failure.

3. In 6 of 7 patients with proteinuria, the amount of albumin degraded was significantly less

than in non-proteinuric subjects and the amount of protein synthesized was approximately the same as in subjects without proteinuria.

4. A subject with myxedema but without proteinuria had the greatest quantity of exchangeable albumin, the largest extravascular fraction of total exchangeable albumin, and the lowest rate of albumin synthesis.

ACKNOWLEDGMENTS

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OBSERVATIONS CONCERNING THE PRODUCTION AND EXCRETION OF CHOLESTEROL IN MAMMALS. XVI. THE RELATIONSHIP OF THE LIVER TO THE CONTENT AND CONTROL OF PLASMA CHOLESTEROL ESTER[1]

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(Submitted for publication February 4, 1955; accepted May 12, 1955)

Earlier studies of this series (1, 2), and also those of Gould and his associates (3), have demonstrated that the liver serves as the chief source of plasma total cholesterol in the intact animal. This same organ also functions to remove an excess of cholesterol when present in plasma (4–7). Thus, whether the cholesterol content of plasma is experimentally reduced or increased, the liver functions to restore the physiological *status quo*. Only severe interference, therefore, with the hepatic function or perhaps drastic alteration of the physico-chemical integrity of plasma cholesterol itself (8, 9) can prevent such restoration.

Despite this certainty that the liver serves as the indispensable source of plasma cholesterol, it has never been ascertained whether this organ discharges only free cholesterol of which a large portion will be esterified elsewhere or whether it supplies both free and conjugated forms to the plasma. It has been tacitly assumed, however, ever since Epstein's observation (10) of the dearth of esterified cholesterol in the blood of patients suffering from severe liver disease, that the liver is probably the chief site of esterification of plasma cholesterol.

In view of this seeming absence of experimental data directly bearing upon the origin and fate of plasma cholesterol ester, the present series of experiments designed to investigate this question was undertaken. The results of these experimental observations appear to indicate that when the plasma content of esterified cholesterol is acutely reduced in the experimental animal, the liver serves as the chief, if not the indispensable source of its replacement.

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I. THE ROLE OF THE GASTRO-INTESTINAL TRACT (INCLUDING THE PANCREAS) IN RESTORATION OF PLASMA CHOLESTEROL ESTER

The presence of a potent cholesterol esterase in pancreatic and intestinal fluid and the known intestinal absorption of dietary cholesterol predominantly in the ester form (11, 12) present the possibility that the chief source of plasma cholesterol ester stems from the gastro-intestinal tract. Conceivably such ester could be received by the blood via the intestinal lymph (13) or perhaps directly from the blood traversing either the pancreas or the intestinal mucosa. The following experiment was done to determine this possibility.

A. *Methods*

(a) *Total extirpation of the gastro-intestinal tract (including the pancreas and spleen) following experimental reduction of plasma total and cholesterol ester.* A series of seven anesthetized rats were subjected to plasmapheresis (2) following which the stomach, the small and large intestines, the pancreas and spleen were removed. Blood samples obtained before, immediately and 24 hours after plasmapheresis were analyzed for their total and esterified cholesterol by previously described (14) methods.

(b) *Control animals.* A series of 10 rats were subjected to plasmapheresis for control purposes. Blood samples obtained immediately before and immediately, 12 and 24 hours, after plasmapheresis were analyzed for their total and esterified cholesterol content.

B. *Results*

Despite the total absence of their entire gastro-intestinal tract, including the pancreas, the experimental rats were able to restore their plasma cholesterol ester content after it had been reduced by plasmapheresis.

Thus, despite the fact that their average cholesterol ester content had been reduced from 38.0 to 9.0 mg. per 100 cc. by the plasmapheresis (see

Table I A), 12 hours later, it had again risen to 33.0 mg. per 100 cc., a rise approximately equal to that found in the intact control rats (see and compare Table I A and I D).

These results left no doubt that replacement of acutely lost plasma cholesterol ester was a process which could proceed independently of the absorption of dietary derived cholesterol and also of the possible esterifying mechanisms present in the pancreas and intestinal mucosa.

II. THE ROLE OF THE KIDNEY IN THE RESTORATION OF PLASMA CHOLESTEROL ESTER

Since removal of the rat's kidney was found to alter the plasma cholesterol content (15), it seemed desirable to determine the possible role of this organ in the restoration of the plasma cholesterol ester level previously reduced by plasmapheresis.

A. *Methods*

Six male rats were plasmapheresed as above and then both kidneys were removed. Blood samples obtained before, immediately, and 24 hours after the plasmapheresis were analyzed for total and esterified cholesterol content.

B. *Results*

Despite the absence of kidneys, these rats appeared able to restore their lost plasma cholesterol ester as readily as the intact control animals. The average plasma ester content which was reduced from 38 to 16 mg. per 100 cc. (see Table I B) returned to 34 mg. per 100 cc., 24 hours later.

III. THE ROLE OF THE LIVER IN THE RESTORATION OF PLASMA CHOLESTEROL ESTER

In an earlier study of this series (2) we had observed that in the absence of sufficient hepatic tissue, the rat was not able to restore either its plasma free or esterified cholesterol if both had been previously reduced by plasmapheresis. While this study indicated that the liver was indispensable for ester replacement, it did not demonstrate necessarily that the esterification actually occurred in the liver. In other words, it can be

TABLE I

The effect of various procedures upon replacement of plasma cholesterol after experimental removal

No. of rats	Av. wt. (gm.)	Amt. blood plasmapheresed cc.	Plasma cholesterol (mg./100 cc.) Before plasmapheresis Total	Before plasmapheresis Ester	Immed. after plasmapheresis Total	Immed. after plasmapheresis Ester	12 hrs. after plasmapheresis Total	12 hrs. after plasmapheresis Ester	24 hrs. after plasmapheresis Total	24 hrs. after plasmapheresis Ester
A. Eviscerated Rats										
7	285 (230–334)*	27 (23–30)	46 (38–60) ±2.6†	38 (33–43) ±1.6	11 (2–18) ±1.9	9.0 (2–16) ±1.7	68 (52–80) ±3.0	33 (28–40) ±1.5		
B. Nephrectomized Rats										
6	240 (210–265)	27 (26–28)	48 (40–55) ±2.0	38 (37–42) ±1.3	18 (12–24) ±1.7	16 (11–22) ±1.6			50 (47–53) ±3.2	34 (31–39) ±2.8
C. Partially Hepatectomized Rats Given Free Cholesterol after Plasmapheresis										
8	264 (226–316)	25 (20–28)	49 (40–68) ±2.9	41 (28–58) ±2.8	46 (37–72) ±4.1	25 (16–34) ±2.2	43 (30–57) ±2.9	20 (7–34) ±3.3	43 (26–59) ±3.6	23 (7–42) ±4.2
D. Normal Rats—Control										
10	243 (209–300)	26 (22–32)	54 (44–61) ±1.7	40 (33–48) ±1.4	20 (10–31) ±2.0	16 (8–31) ±1.9	61 (46–81) ±6.3	36 (30–42) ±2.2	54 (44–60) ±2.4	40 (27–45) ±3.0

* Range of values.
† Standard error of the mean.

postulated that the liver may discharge *only* free cholesterol which then is subsequently esterified elsewhere.

In order to distinguish the possible role of the liver in both of these processes, it would be necessary to administer to an animal with insufficient hepatic tissue, a sufficient amount of cholesterol in the free state and then determine whether esterification of such cholesterol occurred despite inadequate liver function. This type of experiment was done in the following manner.

A. *Methods*

(a) *Subtotal hepatectomy following experimental reduction of only plasma cholesterol ester.* Eight rats were anesthetized and approximately 60 to 70 per cent of their liver was removed by a previously described technique (1). Removal of this amount of liver tissue is sufficient to prevent any restoration of lost total and esterified plasma cholesterol irrespective of the severity of the loss of cholesterol (2). Immediately following the hepatectomy, the animals were subjected to the plasmapheresis process as done above except that the resultant loss in total cholesterol was overcome by the intravenous injection of a specially prepared hypercholesteremic serum containing predominantly free cholesterol (approximately 140 mg. of free cholesterol per 100 cc.). This procedure left the rat with a markedly reduced ester but a proportionately increased increment of free cholesterol.

The hypercholesteremic serum was obtained by bleeding and collecting the serum of rats which had been injected 15 minutes previously with 2.5 cc. of a 2 per cent emulsion of cholesterol prepared as described in a previous study (16). In this latter study, it was found that after injection of this emulsion into a rat, approximately 60 to 70 per cent of the injected cholesterol disappeared immediately from the circulating blood but that increment of excess cholesterol remaining in the blood 15 minutes after injection exhibited a slow rate of disappearance comparable to that observed when cholesterol in soluble lipoprotein form is administered (4). This cholesterol also appeared to be esterified by the injected rat (16). It therefore seems probable that this remaining cholesterol is in soluble, not particulate form. It was found necessary to administer this type of hypercholesteremic serum rather than that produced by cholate (15) or Triton (9) administration because such sera invariably contain a plethora of esterified as well as free cholesterol.

Blood samples obtained before, immediately, 12 and 24 hours later were analyzed for total and ester cholesterol content.

No control animals with intact livers were administered the hypercholesteremic serum because, as already demonstrated (see Table I D), they were able to replace their lost ester without the necessity of administering free cholesterol as a possible precursor.

B. *Results*

Despite the fact that the eight partially hepatectomized rats after plasmapheresis were supplied with additional free cholesterol amply sufficient for its possible esterification by extra-hepatic sources, they still were found unable to replace the esterified cholesterol lost by plasmapheresis. As Table I C demonstrates, although the total cholesterol had not been reduced after plasmapheresis because of the subsequent injection of excess free cholesterol, the average plasma ester concentration reduced by plasmapheresis to 24.0 mg. per 100 cc. actually declined further to 20 and 23 mg. per 100 cc. at 12 and 24 hours, respectively, after plasmapheresis. Failure of the free cholesterol increment to fall during this same period also indicates that it was undoubtedly in a soluble, not particulate, state or else it quickly would have been withdrawn from circulation by the R-E system (6).

IV. THE ROLE OF THE GASTRO-INTESTINAL TRACT AND THE LIVER IN THE REMOVAL OF EXCESS CHOLESTEROL ESTER

A. *Methods*

(a) *Total extirpation of the gastro-intestinal tract (including pancreas and spleen) followed by injection of cholesterol ester.* Five male rats (av. wt.: 319 grams) were eviscerated as described above and then each was given approximately 12 mg. of cholesterol ester in a 1.2 per cent emulsion. This emulsion was prepared by dissolving cholesterol acetate in 1 volume of warm "Sorlate" [2] and diluting with 3 volumes of 0.85 per cent NaCl solution to yield an emulsion containing 1.2 per cent cholesterol. Blood samples obtained immediately, three and six hours after injection were analyzed for total and ester cholesterol.

A series of 10 normal rats were injected for control purposes with 12 mg. of the cholesterol ester and blood samples obtained before, immediately, 3, 6, 12 and 24 hours after injection were analyzed for total and ester cholesterol.

(b) *Total hepatectomy followed by injection of cholesterol ester.* A second series of five rats were similarly eviscerated but also functionally hepatectomized by ligating their hepatic artery. They also were injected with the same amount of ester cholesterol. Blood samples obtained immediately and three hours after injection were analyzed for total and ester cholesterol.

[2] Sorbitan monoleate polyoxyethylene (Abbott).

B. *Results*

The normal intact rat appeared able to rid its plasma as easily of the pure cholesterol ester as of a quantitatively comparable mixture of free and ester cholesterol (4). Thus in three hours, the average ester content had fallen from 244 to 159 mg. per 100 cc. (see Table II A), and at the end of six hours, to 65 mg. per 100 cc. A similar fall in three hours was observed in the eviscerated rats (see Table II B) and the moderate lag observed at the end of six hours might have been due to the rather poor condition of these rats at this time.

The considerable fall observed in the above two series, however, was not observed in the hepatectomized rats (see Table II C). The amount of ester found at the end of three hours (260 mg. per 100 cc.) was about the same as that present immediately after its injection. Thus, the liverless animal appears unable to rid its plasma of excess esterified cholesterol.

DISCUSSION

The data presented above demonstrate that restoration of plasma cholesterol ester lost by plasmapheresis can be effected by the eviscerated or nephrectomized animal as rapidly as by the intact animal. This suggests a total independence of the control of plasma cholesterol ester level from intestinally derived cholesterol ester. On the other hand, the failure of similar restoration of reduced plasma cholesterol ester in the partially hepatectomized animal, even when the latter is given an excess amount of free cholesterol as a possible precursor source for esterified cholesterol, strongly suggests that the liver serves as the chief, if not the indispensable source of plasma cholesterol ester. It would seem possible that the blood cholesterol esterase system described by Sperry, Brand, and Stoyanoff (17–20) might be active in restoring a reduced plasma cholesterol ester concentration to normal levels. No such restorative ability was observed in our experiments, either because the rat lacks such a system (21) or because its action is not quantitatively significant under our experimental conditions. The experimentally derived data offer an explanation for the frequently observed precipitous fall in plasma cholesterol ester component of patients suffering from severe parenchymatous hepatic disease (10).

These observations attesting as they do to the indispensability of the liver in the origin of plasma

TABLE II

The rate of disappearance of injected cholesterol ester

No. of rats	Av. wt.	Plasma cholesterol (*mg./100 cc.*)							
		Before injection		Immed. after injection		Three hrs. after injection		Six hrs. after injection	
		Total	Ester	Total	Ester	Total	Ester	Total	Ester
A. Normal Rats—Control									
10	210	50 (36–63)* ±2.4†	41 (28–55) ±2.34	266 (142–319) ±14.5	244 (123–294) ±14.4	168 (115–204) ±12.0	159 (138–186) ±9.4	114 (89–149) ±4.9	64 (45–76) ±3.4
B. Eviscerated Rats									
5	319			233 (192–272) ±16.0	217 (180–316) ±17	196 (158–250) ±19.5	155 (120–200) ±14.8	132 (122–142) ±7.1	91 (88–94) ±4.5
C. Eviscerated-Hepatectomized Rats									
5	206			283 (258–302) ±4.0	268 (243–291) ±9.3	298 (266–397) ±10.4	260 (234–281) ±8.6		

* Range of values.
† Standard error of the mean.

cholesterol ester do not on the other hand necessarily indicate that at any given time, some small portion of this ester may not be derived from extra-hepatic sources including the intestine or blood itself. As was shown in an earlier report (5), the blood of the intact rat is constantly receiving via the thoracic duct, some esterified cholesterol from the intestine. However, not only is such receipt a slow and gradual one but it is probable that much cholesterol ester is travelling enveloped in chylomicra (22) and because of this is quickly removed by the hepatic R-E cells for subsequent transfer to the hepatic parenchymal cells (6). This probable removal of chylomicronous ester cholesterol by the hepatic R-E cell explains why the oral administration of C^{14} cholesterol is not followed by the appearance of significant amounts of radioactive ester in plasma (23), despite the probable absorption of most of the radioactive cholesterol in the esterified state (11, 12).

SUMMARY

1. The rate of replacement of an experimentally reduced plasma ester cholesterol content in the rat was found to be unaffected by total removal of the entire gastro-intestinal tract (including the pancreas) and the kidneys.

2. Cholesterol ester replacement, however, was not found to occur in the drastically hepatectomized rat even when an excess of free cholesterol was administered to the animal.

3. Disappearance of injected excess esterified cholesterol occurred only in the presence of functioning liver tissue.

4. On the basis of these observations, it is concluded that the liver serves as the chief organ both for the supply and subsequent withdrawal of plasma ester cholesterol, and in so doing controls the latter's concentration in plasma at any given instant.

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THE INFLUENCE OF ACTH, CORTISONE, AND HYDROCORTISONE ON THE DISTRIBUTION AND PERIPHERAL METABOLISM OF THYROXINE [1]

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Renewed interest in thyroid-adrenal interrelations has followed recent demonstrations that alterations in the availability of certain adrenocortical steroids may induce pronounced changes in the metabolism of iodine (1–9). These changes have been variously ascribed to effects of cortical steroids on the pituitary gland (1), the thyroid (10, 11) and the kidney (12, 13). Resolution of the precise modes of action of adrenocortical steroids is not yet complete. Nevertheless, it remains of clinical interest that prolonged administration of ACTH or cortisone may produce in man both laboratory findings and a clinical state suggestive of hypothyroidism (3).

It is generally agreed that alterations in the requirements of peripheral tissues for thyroid hormone induce sequential changes in the plasma's concentration of thyroxine and in the thyroidal metabolism of iodine. The present studies were therefore designed to ascertain whether changes in the peripheral degradation of thyroxine participate in the disruption of thyroidal economy induced by cortisone and related steroids. The data obtained indicate that cortisone, hydrocortisone and ACTH, when administered in replacement or in therapeutic doses do not influence the rate of degradation of circulating thyroid hormone.

MATERIALS AND METHODS

Ten patients were studied on a metabolic ward, prior to and during the administration of ACTH or cortical steroids, while receiving constant weighed diets. Eight patients with primary myxedema, maintained in the euthyroid state by means of exogenous thyroid hormone, received average "pharmacologic" doses of cortisone or hydrocortisone (75 to 200 mg. per day). Two moderately hypothyroid patients with panhypopituitarism, receiving no supplemental thyroid hormone, were given 25 mg. of cortisone daily. The control rate of degradation of thyroxine was determined by means of a method employing radioactive thyroxine [4] and described in detail in an earlier communication (14). In accord with their euthyroid clinical status and BMR's, patients with treated myxedema displayed values for the daily degradation of thyroxine within the range of normal for the method employed (14) (Table I). Sufficient time was allowed to elapse following the control test to permit the concentration of radiothyroxine in the serum to decline to negligible values. Administration of ACTH, cortisone or hydrocortisone was then begun according to the dosage schedule listed in Table I. Following a 4 to 5-day period of equilibration, a second determination of the rate of degradation of thyroxine was begun. Administration of hormone was continued throughout the second period of study. Thus, each patient was subjected to a treatment period of at least 14 days. A profound and persistent reduction in circulating eosinophiles was uniformly demonstrated in the myxedematous patients, and was considered an index of the efficacy of ACTH or steroid therapy. In patients with panhypopituitarism, cortisone in daily doses of 25 mg. resulted in moderate reduction of the number of circulating eosinophiles. Serial measurements of serum precipitable iodine (SPI) were made during both test periods, according to the method of Barker, Humphrey, and Soley (15).[5]

RESULTS

Statistical significance of results obtained in individual patients was analyzed according to the "paired-t" test, as described by Snedecor (16).

In only one patient (G. S.) did the administration of ACTH or cortical steroid induce an alteration (decrease) as great as 1 μg. per cent in serially determined values of SPI (Table I). In

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[5] Performed by Bioscience Laboratories, 2231 Carmelina Ave., Los Angeles 64, Calif.

TABLE I

The effect of cortisone, hydrocortisone, and ACTH on the peripheral metabolism of thyroxine *

Pt.	Diagnosis	Treatment (daily dose)	TDS (*Liters*) 1†	TDS (*Liters*) 2†	k (*%/day*) 1	k (*%/day*) 2	V (*ml./day*) 1	V (*ml./day*) 2	SPI (*μg. I %*) 1	SPI (*μg. I %*) 2	D (*μg. I/day*) 1	D (*μg. I/day*) 2	ETT (*μg. I*) 1	ETT (*μg. I*) 2	Body weight (*Kg.*) 1	Body weight (*Kg.*) 2
Cortisone																
M. B.	Panhypopituitarism	25 mg. p.o.	11.6	9.0	7.14	8.45	828	761	3.0	3.5	24.7	26.6	348	315	73.0	71.8
R. R.	Panhypopituitarism	25 mg. p.o.	8.6	8.4	7.53	6.55	648	550	1.9	2.2	12.3	12.1	163	185	65.9	65.9
V. M.	Myxedema	75 mg. p.o.; thyroid gr. 1	7.0	6.5	10.83	11.55	758	751	4.7	4.8	35.6	36.0	329	312	46.8	46.8
M. F.	Myxedema	100 mg. p.o.; thyroid gr. 3	9.8	8.7	9.76	10.04	956	873	6.3	6.8	60.2	59.4	617	592	56.4	55.5
P. C.	Myxedema	200 mg. p.o.; thyroid gr. 2	7.9	6.3	10.19	13.10	805	825	4.8	5.3	38.6	43.7	379	334	58.2	57.7
Hydrocortisone																
E. G.	Myxedema	75 mg. p.o.; thyroid gr. 2	11.7	11.9	10.66	9.90	1,247	1,178	3.2	3.4	39.9	40.1	375	404	44.5	44.5
M. McC.	Myxedema	75 mg. p.o.; sodium-L-thyroxine 0.3 mg.	8.1	6.7	10.83	12.16	878	815	9.0	9.8	79.0	79.9	729	657	66.4	66.8
L. McK.	Myxedema	75 mg. p.o.; thyroid gr. 2	7.0	7.2	9.76	10.83	683	780	6.0	5.3	41.0	41.3	420	382	60.5	61.4
ACTH																
G. S.	Myxedema	80 mg. i.m.; thyroid gr. 1.5	9.5	7.7	9.00	11.95	855	920	4.4	3.4	37.6	31.3	418	262	52.7	56.8
A. W.	Myxedema	80 mg. i.m.; thyroid gr. 3.5	9.2	8.3	11.74	13.33	1,080	1,107	6.4	6.5	69.2	72.0	589	540	60.5	60.9
		Mean	9.0	8.1	9.74	10.79	874	856	5.0	5.1	43.8	44.2	437	398	58.5	58.8
		P Value	<.01		<.05		>0.5		>0.5		>0.5		.05		>0.2	

* TDS—Thyroxine distribution space. Mean normal value, 9.4 (14).
k—Fractional rate of turnover of radiothyroxine. Mean normal value, 10.56.
V—Daily volume turnover (TDS × k). Mean normal value, 1000.
D—Daily degradation of thyroxine (SPI × V/100). Mean normal value, 53.6.
ETT—Extrathyroidal thyroxine (SPI × TDS × 10). Mean normal value, 508.

† 1—Control test.
2—Test during administration of ACTH or cortical steroid.

eight of ten patients, SPI increased during the experimental period. These changes, however, did not prove to be statistically significant.

The administration of ACTH or cortical steroid was accompanied by a statistically significant reduction in the volume of distribution of thyroxine ($p < .01$), and a significant increase in its fractional rate of turnover ($p < .05$). The volume of the "thyroxine space" diminished in eight of ten patients, in seven of whom the fractional rate of turnover of radiothyroxine was augmented. As a result of the generally reciprocal changes in these functions, the daily volume turnover, *i.e.*, the volume of the thyroxine space whose hormone was replenished daily, remained essentially unchanged. The only changes in volume turnover which exceeded 10 per cent were an increase of 14 per cent in patient L. McK. and a decrease of 15 per cent in patient R. R.

A reduction in the total quantity of hormonal iodine in extrathyroidal tissues (thyroxine space × SPI) [6] was observed in eight of ten patients. Changes in this function were at the borderline of assumed limits of statistical significance ($p = .05$).

Values for the absolute rate of degradation of thyroxine, calculated as the product of the daily volume turnover and the SPI, were not significantly altered by the administration of ACTH or cortical steroid ($p > 0.5$).

DISCUSSION

In normal animals and man, administration of ACTH or cortisone may result in a diminution of the thyroidal accumulation of I^{131} (1–9), a reduction in thyroidal clearance of radioiodide (17), and a decline in the concentration of circulating thyroid hormone (1, 3, 18–20). These effects alone might produce secondary alterations in the peripheral degradation of thyroxine. For this reason, studies were performed in patients with primary myxedema, in whom a thyroidal effect of cortical steroids could not contribute to changes in the rate of degradation of thyroid hormone. In such patients, constant doses of exogenous thyroid hormone were the sole source of circulating thyroxine. Changes in the SPI, during the administration of ACTH or cortical steroids, would then reflect either an alteration in peripheral degradation or in the volume of distribution of thyroxine. These alternatives could in turn be distinguished by studies of the distribution and rate of turnover of radioactive thyroxine. Thus, effects of cortical steroids could be evaluated by two criteria. The two patients with panhypopituitarism afforded an opportunity to study the effects of restorative or replacement doses of cortisone, under circumstances in which it might be anticipated that central (hypophyseal or thyroidal) effects of the steroid would be minimal.

The results of these studies indicate that under conditions of constant dietary intake, ACTH, cortisone and hydrocortisone may alter the volume of distribution of thyroid hormone, but do not regularly influence its rate of degradation. Thus, reduction in thyroxine space was accompanied by an increase in the fractional rate of turnover of thyroxine. As a result, the daily volume turnover, *i.e.*, the volume of thyroxine space whose hormone was degraded daily, remained unchanged.

Although the daily degradation of thyroxine was not altered, the total quantity of hormone contained in extrathyroidal tissues appeared to decrease. A possible explanation for these findings may be offered. In all patients, hypophyseal or adrenal hormone was administered for several days prior to determination of the rate of degradation of thyroxine. This was done in order to allow equilibration of the patient under the new hormonal regimen. If, as the present data indicate, these hormones induce a reduction in thyroxine space, an increase in SPI would be anticipated. Since within the normal range of SPI, utilization is proportional to the plasma's concentration of hormone (14), an increase in the total quantity of thyroxine degraded would result. This would tend to reduce the SPI toward its initial value. Excessive utilization of thyroxine during the period of contraction of the thyroxine space, would, in the face of a constant supply of exogenous hormone, diminish the total quantity of hormone contained within the extrathyroidal pool.

In only one patient in ten were the findings not consistent with this hypothesis. Patient G. S. demonstrated a diminution in both the thyroxine space and SPI. Despite the diminution in SPI,

[6] In calculating values of the daily degradation and the extrathyroidal thyroxine, serum precipitable iodine was considered to consist solely of thyroxine iodine.

the rate of degradation of thyroxine during the administration of ACTH was as great as it had been during the control period. Others have reported profound diminutions in the SPI of euthyroid patients receiving cortisone for no longer than the period of treatment employed in the present study (2, 18). Furthermore, no abnormality in the peripheral degradation of thyroxine could be demonstrated in a patient with long-standing active Cushing's syndrome.[7] These observations indicate that failure of the SPI to decline during the present study did not result from therapy of insufficient duration.

The present findings are in accord with the observations of O'Neal and Heinbecker, who noted that cortisone induced no alterations in the rate of decline of SPI in the serum of hypophysectomized dogs following the administration of TSH (21). Similarly, Hill, Reiss, Forsham, and Thorn (1), as well as Engstrom and Markardt, have reported that cortisone did not influence the SPI of myxedematous patients given exogenous thyroid hormone (22). No explanation is apparent for the discrepancy between these observations and those of Bondy and Hagewood, who reported that in thyroidectomized rats given thyroxine, the administration of cortisone was accompanied by a marked augmentation of SPI (20).

If, as the present data indicate, cortisone does not alter the peripheral degradation of thyroxine, then reduction in the concentration of circulating thyroid hormone, which frequently follows the administration of this steroid, must be ascribed to other mechanisms. Whether this decline in SPI results from a diminution in the glandular production of hormone, the rate of turnover of the glandular hormonal pool, or both, and whether it is mediated by an action of cortisone on the pituitary gland or directly on the thyroid remains to be elucidated.

SUMMARY

1. The distribution and rate of turnover of radioactive l-thyroxine have been examined in ten patients with primary myxedema or panhypopituitarism prior to and during the administration of ACTH, cortisone or hydrocortisone.

2. As judged by the metabolism of radioactive thyroxine and by measurements of serum precipitable iodine, ACTH and cortical steroids did not alter the rate of degradation of thyroxine.

[7] Values observed: thyroxine distribution space, 9.7 liters; fractional turnover rate, 9.63 per cent per day; daily volume turnover, 934 ml.; SPI, 3.4 μg. per cent; thyroxine degradation, 31.7 μg. iodine per day; extrathyroidal thyroxine, 330 μg. iodine.

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PULMONARY CAPILLARY BLOOD FLOW IN MAN [1, 2]

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Although it is obvious that the blood flow through the pulmonary artery is pulsatile, it is not so certain that it remains so within the pulmonary capillaries. On the contrary, the direct Fick method for measuring cardiac output expresses mean flow per unit time, and calculation of pulmonary arteriolar resistance tacitly assumes the existence of a non-pulsatile blood flow. Furthermore, it is not known whether the rate of gas diffusion from alveolus to capillary varies with the phase of the cardiac cycle.

The present communication describes a method for measuring instantaneous blood flow in the pulmonary capillary bed in man. A continuous recording of pressure within an airtight body plethysmograph is made before and after the inhalation of nitrous oxide. As the gas is absorbed by the blood entering the lung capillaries, the pressure within the body plethysmograph falls. This fall in pressure is proportional to the pulmonary capillary blood flow, and was found to vary according to the phase of the cardiac cycle.

METHOD

An airtight body plethysmograph of approximately 600 liters capacity, previously constructed for the measurement of alveolar pressure (1) was used (Figure 1). The differential pressure between the plethysmograph chamber and an adjacent "compensatory" chamber was measured, using a Lilly capacitance manometer, Brush D.C. amplifier and recorder. This arrangement eliminated artefacts produced by extraneous sounds in the laboratory, alterations in room temperature and changes in ambient barometric pressure. The sensitivity of the recording system was .04 cm. H_2O per cm. deflection, and the response time was satisfactory up to a rate of 35 cycles per second. The pressure between the plethysmograph and compensatory chamber could be equalized by a valve, and the plethysmograph could be vented to the exterior by a solenoid operated valve in the wall of the chamber.

The subjects, who had been active in the laboratory, were permitted to sit for 5 to 15 minutes before entering the plethysmograph. During the test, they were required to breathe in a special way, and to turn valves; they were by no means basal. The subject sat in the closed plethysmograph with two 5-liter bags and two sampling tubes. His electrocardiograph (lead CF.4) was recorded simultaneously with the plethysmograph pressure, using a second Brush D.C. amplifier operating the remaining pen of the Brush recorder. Two minutes were allowed for temperature equilibration to occur, but in spite of this, some slight rise in pressure within the plethysmograph continued owing to continued heating of the contained air by the subject's body. To measure this and also R.Q. effects during the subsequent breath-holding procedures a control record was first obtained. The subject twice exhaled almost to his residual capacity and then took a deep inspiration. After the second inspiration, he exhaled to resting lung volume and held his breath with the glottis open for a few seconds while simultaneous records of plethysmograph pressure and ECG were obtained. In addition to the pressure changes

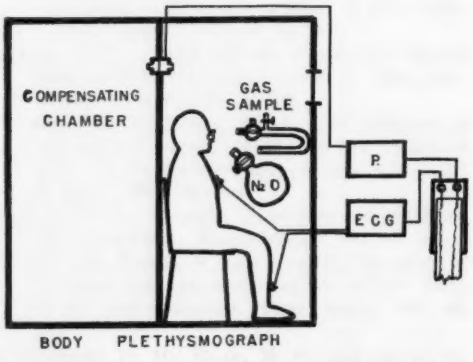

FIG. 1. DIAGRAM OF BODY PLETHYSMOGRAPH, MANOMETER, AND ELECTROCARDIOGRAPH RECORDING SYSTEM

For pictorial clarity, valves between the plethysmograph, compensating chamber, and the exterior are not shown.

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[2] Presented in preliminary form at the Fall Meetings of the American Physiological Society, 1954. DuBois, A. B., and Lee, G. de J., (Am. J. Physiol., In press).

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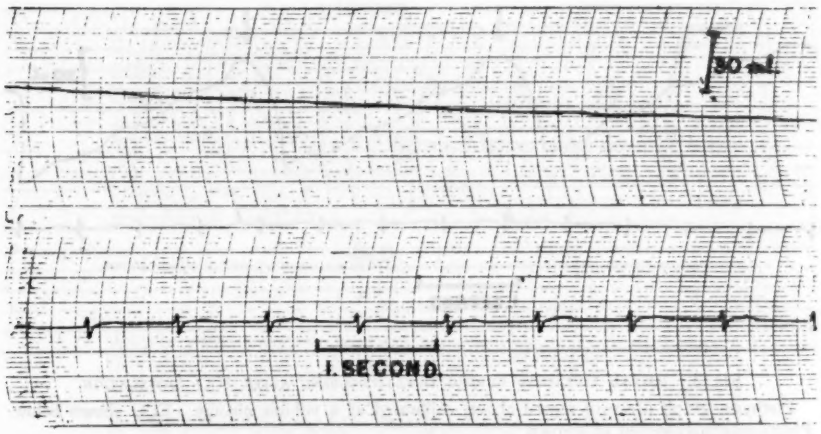

FIG. 2. CONTROL RECORD DURING BREATHHOLDING AFTER AIR INHALATION
Plethysmograph pressure record (inverted) above, calibrated as a volume change. ECG shown below.

occurring from heating and R.Q. effects, small variations in pressure were seen to occur in association with the heart beat (Figure 2).

After a short period of normal breathing, the subject again exhaled almost to his residual volume and then took a deep inspiration from a 5-liter bag containing 100 per cent oxygen. This was done to raise the alveolar oxygen content so that when nitrous oxide was subsequently breathed, the resultant alveolar gas mixture would contain approximately the normal concentration of oxygen. Next, the subject again fully exhaled and took a maximal inspiration of 100 per cent nitrous oxide from a second 5-liter bag. He then exhaled through a Haldane gas sampling tube to resting lung volume and held his breath with the glottis open. The plethysmograph pressure fell in a characteristic fashion as the nitrous oxide was absorbed by the blood entering the lung capillaries (Figure 3). After 5 to 10 seconds breathholding the subject exhaled maximally through a second Haldane gas sampling tube.

The two expiratory samples were taken for subsequent analysis for nitrous oxide in a mass spectrometer. From these samples, the mean alveolar gas concentration during each cardiac cycle was calculated. The subject usually began to feel slightly 'intoxicated' some 15 to 20 seconds after inhaling the gas; this sensation disappeared rapidly after inhaling several breaths of air from the plethysmograph; it was more marked if oxygen was not inspired prior to the nitrous oxide inspiration.

The calculation of mean alveolar gas concentration from expired air samples assumes satisfactory distribution of the inspired gases within the lungs. All the subjects studied were healthy young adults. Under such circumstances it has been shown that the alveolar plateau recorded by a rapid gas analyzer can be extrapolated backward to the dead space (2, 3) and forward to the residual volume (4, 5). Since the mean concentration of alveolar gas is the mean of the integral of the product of concentration and volume over the entire range of lung volume, it is reasonable to assume that the mean alveolar concentration is represented as a point located at approximately mid expired volume on the alveolar plateau in a normal subject.

Expiration into a rapid gas analyzer showed that after a single breath of nitrous oxide, the alveolar gas concentration was approximately 58 per cent N_2O. However, if the breath was held at resting volume for varying intervals of time before exhaling into the analyzer, the alveolar concentration fell progressively at approximately 0.5 per cent N_2O per second. After exercise, the rate of decline was considerably faster (0.7 to 1.8 per cent N_2O per second).

The change in pressure in the plethysmograph was converted to volume change by calibrating with a small volume of air alternately injected and withdrawn, using a 30 ml. syringe or a diaphragm type pump. The calibrating volume under ambient conditions at 26° to 28° C., and 40 to 50 per cent saturated (unpublished measurements by Botelho) was converted to BTPS by an appropriate factor, assuming a barometric pressure of 760 mm. Hg. It produced a deflection of approximately 12 mm. on the record.

Having completed the procedure under resting conditions, the whole procedure was repeated after half a minute of knee-bending exercises in the plethysmograph.

Calculation of blood flow

The solubility of nitrous oxide (S_{N_2O}) in whole blood is 47 vol. per 100 ml. blood per atmosphere at BTPS (6). Since the volume of N_2O (V_{N_2O}) which dissolves in the blood is proportional to the alveolar fraction of N_2O

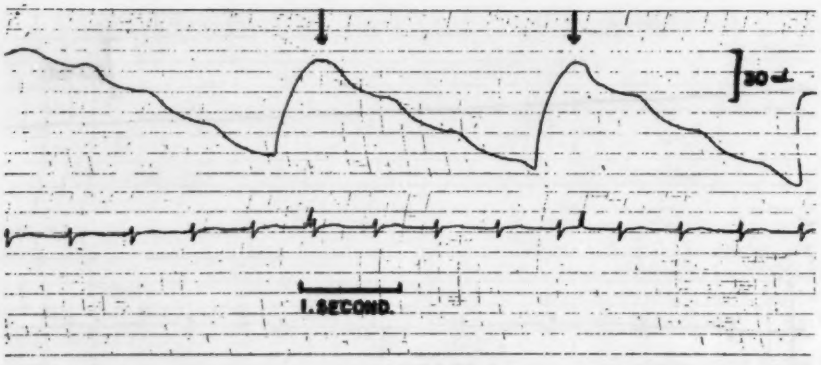

FIG. 3. RECORD OBTAINED DURING BREATHHOLDING AFTER N_2O INHALATION

Plethysmograph pressure record above, calibrated as a volume change. ECG shown below. Plethysmograph vented at arrows.

($F_{A_{N_2O}}$), it is necessary to calculate the latter from the samples which have been analyzed, as the fraction of dry gas (F_{N_2O}), by the formula: $F_{A_{N_2O}} = F_{N_2O} \cdot \frac{B-47}{760}$, where the symbols are standard (7).

The volume of gas absorbed in a given time (t) is also proportional to the quantity of blood (Q) equilibrated with the gas at that time. Combining terms

$$V_{N_2O} = S_{N_2O} \cdot F_{A_{N_2O}} \cdot Q.$$

Rearranging and differentiating with respect to time

$$\dot{Q} = \frac{\dot{V}_{N_2O}}{S_{N_2O} \cdot F_{A_{N_2O}}},$$

where $\dot{Q}$ = cardiac output
$\dot{V}_{N_2O}$ = rate of absorption
S_{N_2O} = solubility in whole blood
} of nitrous oxide

all terms being expressed in liters per minute at BTPS.

Two separate methods for measuring the rate of gas absorption ($\dot{V}$) were employed:

(a) Four consecutive cardiac cycles were measured for pressure deflection at 0.04 second intervals, commencing in time with each R-wave of the ECG. The pressure changes during corresponding intervals of time in each cycle were then averaged to obtain a succession of mean values of V and so eliminate effects of random noise artefact.

The same was done for the control period, and the control values were subtracted from those obtained during the N_2O period in order to eliminate the recurrent variations produced by mechanical gas compression with the heart beat, temperature, and R.Q. changes already mentioned.

(b) The output from the capacitance manometer amplifier recording the plethysmograph pressure (V) was led through a 'differentiating' circuit consisting of a capacitance (C) and resistance (R); the voltage across the resistance was measured by a Brush DC amplifier and recorder. The values of C and R were selected to give a time constant of 0.06 second. The output was approximately proportional to the rate of change of pressure in the plethysmograph and hence proportional to the rate of blood flow (Figure 4).

It was considered that part of the pressure drop in the plethysmograph during nitrous oxide uptake might possibly be due to uptake in the lung tissues themselves, rather than by the blood. This possibility was therefore tested, using the trachea, heart and lungs of dogs, obtained immediately following the termination of other experiments in which the lungs had not been involved. These isolated lungs contained blood that was not flowing. The preparation was placed in a plethysmograph of approximately 25 liters capacity and inflation experiments were made which were identical with the procedure carried out by our subjects in the body plethysmograph. After the first second following inflation of the dog's lungs with 100 per cent nitrous oxide, only minor changes in plethysmograph pressure occurred:

Dog 1: 0.09 ml. per sec. N_2O uptake. Weight of lungs: 176 gm.
Dog 2: 0.13 ml. per sec. N_2O uptake. Weight of lungs: 155 gm.
Dog 3: 0.02 ml. per sec. N_2O uptake. Weight of lungs: 174 gm. (Average: 0.08 ml. per sec. N_2O uptake.)

Assuming that human lungs are approximately four times as heavy as those of the dog, then approximately 0.3 ml. N_2O per sec. would be taken up, due to delayed equilibration. However, the uptake due to blood flow is approximately 30 ml. N_2O per sec., so that the factor of delayed lung tissue equilibration is negligible in the present study on man.

Although N_2O is an inert gas chemically, it seemed possible that it might be evoking a local vasodilator effect upon the lung vessels. This could have led to the production of a pulsatile blood flow within the lung capillaries not necessarily present in the normal state. To test this possibility, the pressures obtained via a catheter

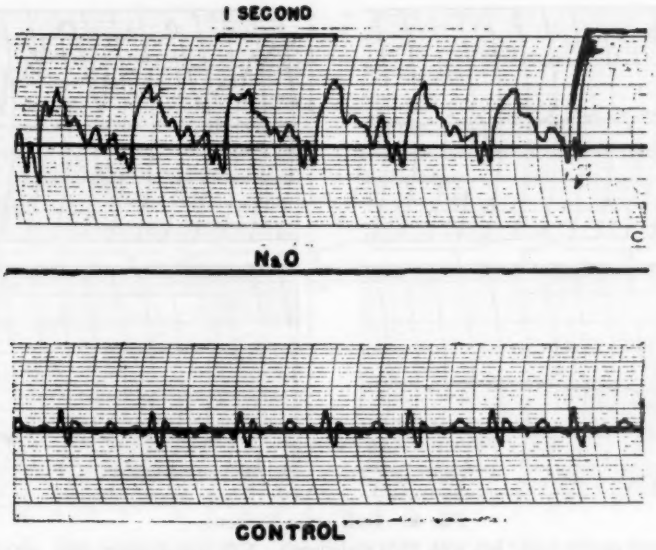

FIG. 4A. G. L. AT RESTING LUNG VOLUME

Control record, on air, is below, and gas absorption record, on N_2O is above.

wedged in the pulmonary vein of three open-chested dogs under light Nembutal® anesthesia were studied. The animals alternately breathed room air and 50 per cent N_2O in oxygen. No alteration in the pressure pulsations was obtained when the N_2O mixture was breathed. This suggested that no change in the physical state of the vessels had occurred.

RESULTS

Instantaneous pulmonary capillary blood flow measurements with simultaneous electrocardiograph records were obtained from five healthy young male subjects sitting at rest following laboratory activity and again immediately after moderate exercise. The results are shown graphically in Figures 5 and 6, where instantaneous flow rates expressed in liters per minute are shown in relation to the electrocardiographic events. In all instances the rate of pulmonary capillary blood flow is seen to vary markedly, being slowest shortly after the QRS complex of the ECG and most

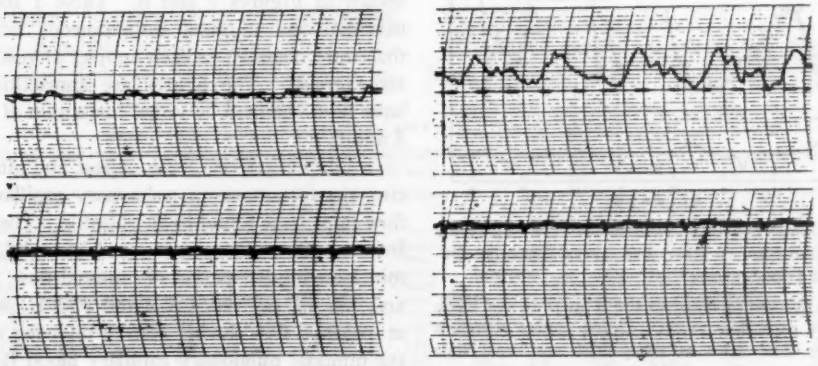

FIG. 4B. DUB IN EXPIRATORY POSITION, GLOTTIS OPEN

Control record, upper left, with ECG beneath. N_2O record, upper right, with ECG below.

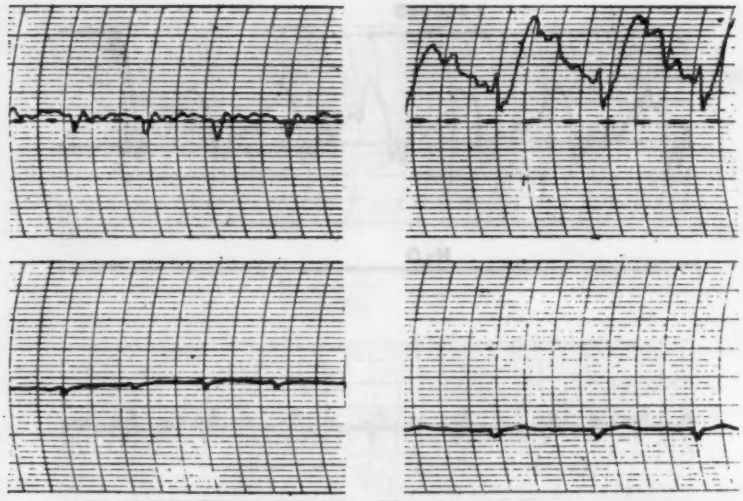

FIG. 4c. DuB AFTER EXERCISE

Control record, upper left, with ECG underneath. N_2O record, upper right, with ECG below.

FIG. 4. "DIFFERENTIATED" PLETHYSMOGRAPH PRESSURE RECORDS

rapid subsequent to onset of the T wave. The closed circles in Figure 5 represent instantaneous flow rate measurements at each 0.04 second following the R wave (zero time) obtained from four consecutive heart cycles (see section on *Calculation of blood flow*). The solid line represents the average instantaneous flow rate of the four cycles. The dashed horizontal line represents the mean flow rate or mean cardiac output. In Figure 6, for the sake of pictorial clarity, only the line representing the average instantaneous flow rates of four cardiac cycles have been shown.

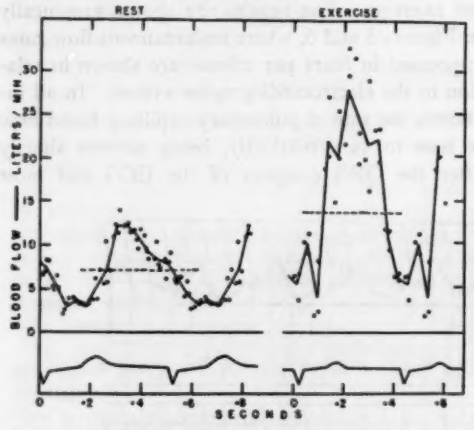

FIG. 5. INSTANTANEOUS PULMONARY CAPILLARY BLOOD FLOW RATES AT REST AND AFTER EXERCISE OBTAINED FROM ONE SUBJECT

Zero time arbitrarily taken as the R wave of the ECG, shown below each flow curve. Closed circles represent instantaneous flow rates at every 0.04 sec. from four consecutive heart cycles. Solid line represents the average instantaneous flow rate obtained from those points. Dashed line represents mean cardiac output per minute.

Sharp transient changes in flow rate occur in the regions at highest and lowest flow rate, as are shown in Figures 5 and 6. Table I shows the maximal and minimal instantaneous flow rates that were obtained in each subject at rest and after exercise. The figures are approximate and have been obtained by visual inspection of Figures 5 and 6.

In all five subjects, both at rest and after exercise, the instantaneous pulmonary capillary blood flow rates were found to be pulsatile. They varied between 40 and 70 ml. per sec. (2.4 and 4.2 L. per min.) during the period of minimal flow, and 203 and 426 ml. per sec. (12.2 and 25.6 L. per min.) at maximal flow during rest. Following exercise, the minimal pulmonary capillary blood flow rates varied between 40 and 128 ml. per sec. (2.4 and 7.7 L. per min.) and the maximal flow rates varied

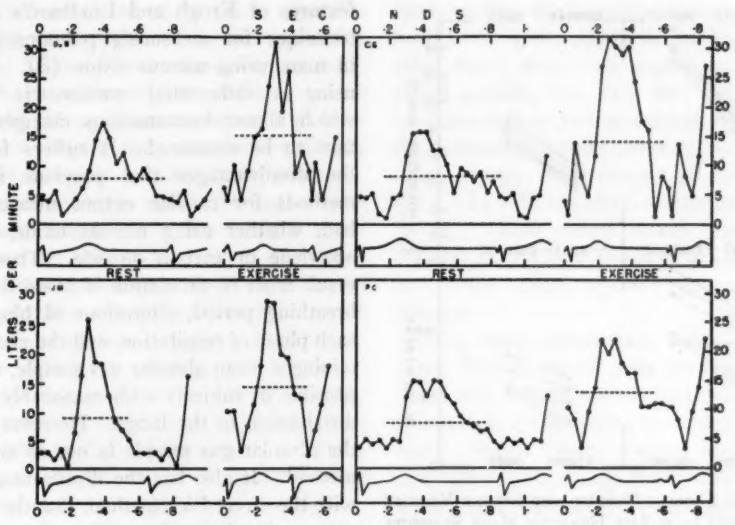

FIG. 6. THE AVERAGE INSTANTANEOUS PULMONARY CAPILLARY FLOW RATE CURVES OBTAINED FROM FOUR CONSECUTIVE CARDIAC CYCLES OF FOUR HEALTHY SUBJECTS AT REST, AND AFTER EXERCISE

Zero time is arbitrarily taken as the R wave of the ECG, shown below each flow curve. The dashed line represents mean cardiac output per minute.

between 370 and 530 ml. per sec. (22.2 and 31.8 L. per min.).

In one individual (subject M) although the mean cardiac output per minute rose after exercise, the instantaneous flow rates per beat remained virtually unaltered. The rise in cardiac output was accomplished in this instance by change in cardiac frequency alone. In the other four subjects, both changes in cardiac frequency and increases in instantaneous pulmonary capillary blood flow rates (stroke output) were responsible for the rise in cardiac output. The ratio of maximal to minimal flow rates in the five subjects varied between 3.6 and 10.7 at rest, and 3.1 and 12.0 after exercise.

Stroke volume measurements can be made from the instantaneous flow curves (Figures 5 and 6), since the area of each curve represents pulmonary capillary blood flow per cardiac cycle. Hence, knowing the pulse rate, or from measurement of the mean slope of the plethysmograph pressure curve during N_2O breathing (Figure 3), the mean cardiac output per minute can be obtained (Figure 7 and Table II).

The mean cardiac output in our resting, nonbasal subjects, was 7.9 L. per min. S.E. ± .26

TABLE I

The maximal and minimal instantaneous pulmonary capillary blood flow rates compared with the mean cardiac output estimations in each of five healthy male subjects before and after moderate exercise

Subject	Rest						Exercise						Mean cardiac output L./min.	
	Cardiac frequency pulse/min.	Minimum ml./sec.	Minimum L./min.	Maximum ml./sec.	Maximum L./min.	Maximum / Minimum	Cardiac frequency pulse/min.	Minimum ml./sec.	Minimum L./min.	Maximum ml./sec.	Maximum L./min.	Maximum / Minimum	Rest	Exercise
C.	72	70	4.2	252	15.1	3.6	97	120	7.2	370	22.2	3.1	7.8	13.1
DuB.	79	43	2.6	290	17.4	6.7	121	128	7.7	526	31.6	4.1	7.8	15.4
G.	72	56	3.4	265	15.9	4.7	97	80	4.8	530	31.8	6.6	8.2	14.2
M.	96	40	2.4	426	25.6	10.7	156	40	2.4	480	28.8	12.0	8.7	14.2
L.	118	55	3.3	203	12.2	3.7	141	88	5.3	466	28.0	5.3	7.1	13.8

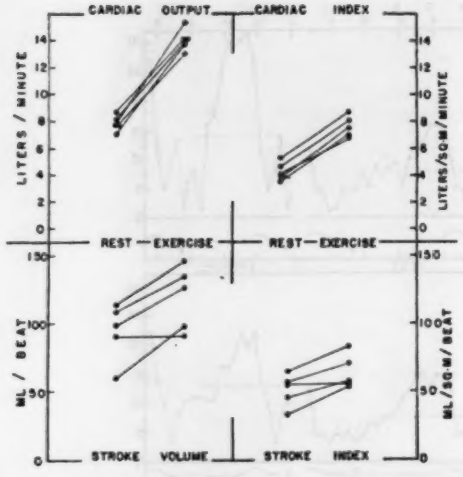

FIG. 7. MEAN CARDIAC OUTPUT AND STROKE VOLUME RESULTS OBTAINED FROM FIVE HEALTHY MALE SUBJECTS BEFORE AND AFTER EXERCISE

(4.3 L. per sq. meter per min.: S.E. ± .32); and after exercise 14.1 L. per min. S.E. ± .37 (7.6 L. per sq. meter per min.: S.E. ± .39).

No simultaneous estimates of cardiac output by other methods were carried out. However, the mean cardiac output by the direct Fick method or by the Hamilton dye injection technique on resting subjects appears to be less than the mean value on these subjects who were actively participating in the procedure.

DISCUSSION

The method here described for measuring pulmonary capillary blood flow incorporate some features of Krogh and Lindhard's single breath technique for measuring pulmonary blood flow in man, using nitrous oxide (8). It differs by using a differential manometric respirometer which allows instantaneous changes in gas uptake to be measured. It suffers from some of the disadvantages that previous indirect Fick methods for cardiac output measurement have had, whether using nitrous oxide, ethyl iodide, acetylene or carbon dioxide. These limitations result from re-circulation of blood during the gas breathing period, alterations of blood flow with each phase of respiration, and the necessity for obtaining a mean alveolar gas sample, which is only possible in subjects with reasonably uniform gas distribution in the lungs. However, accuracy of the alveolar gas sample is not so critical by this method. It also has the disadvantage, compared with the direct Fick method, that the subject must enter a closed chamber, and must be trained in the breathing procedure. Further, no associated data such as pressure measurements, or blood gas samples from various sites in the circulation, can be obtained as with catheter techniques. However it has the advantage of being the only method at present available for instantaneous blood flow in the pulmonary capillaries. Contrasted with it, the dye or isotope dilution methods for measuring cardiac output record the mean flow rate occurring over a period of several heart beats. The present technique allows instantaneous pulmonary capillary blood flow measurements to be made, and also makes possible measurement of the stroke volume of individual right ventricular contractions in man.

TABLE II

Cardiac output and stroke volume data obtained from five healthy male subjects at rest and after moderate exercise

	Rest					Exercise				
Subject	Cardiac frequency *pulses per min.*	Cardiac output *L./min.*	Cardiac index *L./sq. m./ min.*	Stroke volume *ml./beat*	Stroke volume index *ml./sq. m./ beat*	Cardiac frequency *pulses per min.*	Cardiac output *L./min.*	Cardiac index *L./sq. m./ min.*	Stroke volume *ml./beat*	Stroke volume index *ml./sq. m./ beat*
C.	72	7.8	4.1	109	57	97	13.1	6.9	135	71
DuB.	79	7.8	3.5	99	45	121	15.4	7.0	127	58
G.	72	8.2	4.7	114	65	97	14.2	8.1	146	84
M.	96	8.7	5.3	91	56	156	14.2	8.7	91	56
L.	118	7.1	3.9	60	33	141	13.8	7.5	98	54
Mean	87	7.9	4.3	95	51	122	14.1	7.6	119	65
S.D.		.59	.71	13.2	12.4		.83	.87	5.0	3.6
S.E.		.26	.32	5.9	5.5		.37	.39	2.2	1.6

Two qualifications need to be made when interpreting flow data indicated by nitrous oxide uptake in the lungs. Firstly, since nitrous oxide diffuses with approximately the same speed as carbon dioxide, the equilibration between alveolar N_2O and blood entering the pulmonary capillaries is theoretically complete long before the equilibration for oxygen, probably within the first 1/20th of the capillary length (6). The method therefore measures the rate of blood flow at the arteriolar end of the pulmonary capillary only, and gives no information about the blood flow at the venous end of the system. The second qualification concerns pulmonary ventilation-perfusion relationships. A region of lung receiving no ventilation, or presenting an impervious diffusion barrier, will absorb no N_2O despite the presence of blood flow through that area. Such blood must be considered to be passing through a 'physiological shunt' and is not measured by the method.

The instantaneous pulmonary capillary blood flow curves obtained from the nitrous oxide uptake studies do not show a constant flow rate but are pulsatile, having the form and degree shown in the graphs of our five subjects (Figures 5 and 6). The blood flow tends to increase in pulsatility with exercise. Differential pressures between the pulmonary artery and "capillary" curves in man suggested the likelihood of pulsatile capillary flow (9).

Although the amplitude of pulsatility varied among the different subjects and under different physiological conditions, there were certain features to the curves that were consistent and reproducible. The pulmonary blood flow reached its slowest rate approximately 0.1 second after electrical systole and then rapidly accelerated during the period of the T wave, to reach a peak blood flow approximately twice the mean blood flow. The rate of flow then diminished to reach a minimum rate, which was usually less than half the mean flow rate and approximately a fifth of peak flow rate. The instantaneous flow records show that these events do not occur in a smooth or sinusoidal wave form, but with abrupt angulations. Following peak flow there was usually a notch or sharp angulation in the curve; similarly, during the period of minimum flow there were sharp transient changes in the instantaneous flow rate. These sharp changes may be due to artefacts resulting from mechanical compression by the heart of the gases within the lungs (10). On the other hand, since they appear so consistently it seems possible that they may represent real alterations in flow rate associated with the notch in the pulmonary arterial pressure record at the time of pulmonary valve closure in the first instance (11), and with left atrial systole in the second instance. These events would produce transient changes in pressure gradient across the capillary and hence affect capillary flow rates at these instants.

Since many calculations which use pulmonary blood flow as one of their parameters tacitly assume that the pulmonary capillary flow rate is constant, it is interesting to review these and consider whether errors may be introduced by the assumption of a steady flow. The following sections of the discussion are speculative rather than experimental.

Gas exchange

The concepts of ventilation and perfusion, and the ratio $\dot{V}/\dot{Q}$ presume a steady ventilation and steady blood flow for convenience of calculation. Calculation of the variation of alveolar gas tensions during the respiratory cycle (12) shows that under normal conditions and during the steady state it is satisfactory to assume a steady ventilation. The exceptions to this are that the alveolar gas concentrations during a single respiratory cycle and during sudden changes in ventilatory rate do not fluctuate along the same line as the $\dot{V}/\dot{Q}$ ratio. A superficial examination of the effects of pulsatile blood flow will reveal much the same state of affairs. Exchange of oxygen and carbon dioxide between the capillary blood and alveolar gas should mostly take place during the rapid phase of blood flow (systole) and very little during the slow phase (diastole). This intermittent exchange would result in a halting type of progression round the loop describing fluctuations of P_{O_2} and P_{CO_2} on the O_2–CO_2 diagram during a single respiratory cycle (12). Since the respiratory loop itself causes little effect on the $\dot{V}/\dot{Q}$ ratio, intermittent progression round the loop should have little additional effect. In other words, under most circumstances it is satisfactory to assume a constant pulmonary blood flow as far

as the $\dot{V}/\dot{Q}$ ratio is concerned. Again, the exception occurs when rapid changes in alveolar gas concentration are measured. Under such circumstances, instead of a smooth and continuous change in gas concentration as previously supposed, one would expect to find changes occurring in an intermittent fashion. It is of interest that such an intermittency of concentration change has already been noted by Bartels, Severinghaus, Forster, Briscoe, and Bates from continuous analysis of the expired air for O_2, CO_2, and N_2, using a rapid gas analyzer (13).

Analysis of the alveolar-arterial oxygen gradient is based on the $\dot{V}/\dot{Q}$ ratios discussed above. With the presence of a pulsatile capillary blood flow it is possible to conceive of a situation where an A-a gradient, not present in a steady system, could exist. This would exist where the diffusing capacity for oxygen were such that the systolic spurts of blood flow would pass through the capillaries too rapidly to become saturated and where the diastolic pauses could not make up for the systolic rush. Here saturation would be reached at the mean flow rate, and not improved by a slow diastolic flow rate. Such a situation would require that various elements of the stroke output stay in the capillary system unequal lengths of time. This has not been determined experimentally as far as we know.

Hemodynamics

There is no theoretical reason to challenge the direct Fick method for cardiac output measurement during the steady state on the grounds of pulsatile blood flow, but it is interesting to note that the mean blood flow actually exists during only two brief instants of the cardiac cycle during the rise and fall of the instantaneous flow curve. To test the method further, simultaneous direct Fick and plethysmograph measurements of cardiac output need to be made. In the present investigation, the phase of respiration at which the breath was held was seen to influence the stroke volume, which was greatest during inspiration and least during the expiratory phase. In preliminary experiments, the Valsalva maneuver was studied and was found to be associated with a marked increase in stroke volume during the period of the hypertensive overshoot. This is not in agreement with the findings of others who reported a fall in cardiac output during the overshoot period, using the dye technique (14). The stroke volume events associated with the Valsalva maneuver are a gross example of the unsteady state. Calculations were made from constantly timed blood samples, with the result that a disproportionately large concentration of dye was collected during the overshoot period when the stroke volume was large. With the plethysmograph method, nitrous oxide uptake is entirely dependent on flow. The importance of obtaining constant volume samples rather than constant time samples in any modification of the Fick principle for cardiac output measurement is thus well demonstrated in situations where the unsteady state exists (15, 16).

The combination of the elasticity of the arteries and the resistance of the arterioles is capable of converting the pulsatile output of the left ventricle into a constant capillary blood flow in the systemic vascular system. However, the findings here reported regarding pulmonary capillary flow indicate that, although the elasticity of the pulmonary arteries maintains a certain diastolic capillary flow, the combination of this elasticity and the pulmonary arteriolar resistance is not sufficient to induce a constant capillary blood flow. With techniques now available it should be possible, from simultaneous pressure flow data, to separate the factors of elasticity, resistance, and inertia in the pulmonary arterial system. In the absence of such information, it seems likely that the ratio of mean pressure to mean flow commonly used to express "pulmonary arteriolar resistance" will include components of elasticity and inertia. We believe that it would be more logical to calculate the "pulmonary impedance" until the elasticity and inertia factors are computable.

In a pulsatile system it is difficult to conceive of a single value for pulmonary capillary blood volume during all phases of the blood flow, unless the pulmonary capillary inflow exactly matches outflow in time and degree. If this were the case, the oxygen diffusing capacity would tend to decrease with exercise (pulsatile variation of the A-a gradient), the reverse in fact being the case. It would seem more likely that in such a system the pulmonary capillary volume would be capable of increasing to accommodate sharp increases in flow, with the result that the transit time through the

alveolar capillary would remain approximately constant. Exercise would then be associated with an increase in oxygen diffusing capacity, as has been shown to be the case (17–19). The change in pulmonary capillary volume could occur by passive dilatation of already patent vessels as flow increased. Alternatively, the capillary volume could increase by the opening up of previously closed capillaries as flow rate increased and the pressure within the vessels exceeded a certain critical value. Regulation of pulmonary capillary volume by such a critical opening mechanism is an attractive hypothesis (20, 21). Maintenance of a constant pulmonary artery pressure in the face of large and rapid flow changes would become explicable on a physical basis, and the apparent lack of neurogenic control of pulmonary vascular tone would become less perplexing. In addition, opening of fresh vessels at high flow rates would allow both capillary transit time and the diameter of each open capillary to remain constant, with the result that conditions of gas diffusion would remain optimal at all flow rates. Microscopic observation showed opening or closing of lung capillaries in the anesthetized cat (22) and also various types of blood flow, sometimes pulsatile, sometimes steady.

In summary, conditions of pulsatile flow may be expected to produce certain hemodynamic and gas exchange effects which would be absent if the pulmonary capillary blood flow were constant as has sometimes previously been assumed.

SUMMARY

1. A method is described for measuring instantaneous pulmonary capillary blood flow in man.

The subject sat in a body plethysmograph and the plethysmograph pressure was continuously recorded during breathholding after inspiration of nitrous oxide. The plethysmograph pressure fell as the gas was absorbed by the blood entering the pulmonary capillaries. The rate of uptake of nitrous oxide so measured was proportional to the pulmonary capillary blood flow.

2. Observations were made on five healthy male subjects before and after moderate exercise. Pulmonary capillary blood flow was not steady but pulsatile.

Following electrical systole, there was a rapid acceleration of flow to a rate about twice the mean cardiac output, followed by a more gradual decline to low rates of flow. The cycle was repeated with each heart beat.

3. The possible effects on gas exchange and upon the hemodynamics of the pulmonary circulation are discussed.

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PERSISTENCE AND UTILIZATION OF MATERNAL IRON FOR BLOOD FORMATION DURING INFANCY [1, 2]

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The concentration of hemoglobin in the blood of normal infants falls progressively during the first 4 to 6 weeks following birth. The decline results primarily from reduced hematopoiesis, though iron is present for the manufacture of hemoglobin and replacement of erythrocytes. The slow rise in hemoglobin concentration which occurs during the remainder of infancy may be affected by several factors, including the rapidity of body growth, the amount and absorption of dietary iron, and the amount and conservation of iron which was obtained transplacentally (1). An opportunity to measure the persistence and utilization of transplacental iron during infancy arose as a by-product of investigations of maternal red cell volume and hematocrit recently published from this hospital (2, 3).

MATERIAL AND METHODS

In the original studies (2, 3), radioactive iron (Fe^{59}) prepared in the Massachusetts Institute of Technology cyclotron was administered as donor red cells to 11 women on four to seven occasions during pregnancy. From these 11 pregnancies and from five subsequent ones of four of the women, a total of 16 infants were available for study. The mothers and their infants were similar in diet, hygiene and general health to the random population of any obstetrical hospital or well-baby clinic in this area. Figure 1 shows the hemoglobin concentrations of the entire group of infants in comparison with controls from such clinics.

The total activity of Fe^{59} administered as tagged erythrocytes to each pregnant woman ranged from approximately 2 to 12 microcuries, equivalent to an initial dose rate of 0.1 to 1.0 millirad [3] per day in the mother's blood stream. The amount of radiation received by these infants was calculated according to the procedure described by Peacock, Evans, Irvine, Good, Kip, Weiss, and Gibson (5) on the basis of the experimentally measured radioactivity of the circulating hemoglobin iron. The cumulative radiation dose to the blood of the infants for calculation periods averaging 397 days (range 115 to 580) was an average of 131 millirads (range 30 to 354). The biological significance of these radiation levels is revealed by comparison with the cumulative dose to the blood stream received by the infants as a result of cosmic rays, terrestrial gamma rays and naturally occurring K^{40} in the red cells, during the same observation periods. The total dose rate from these sources is about 0.5 millirad per day, and resulted in cumulative doses averaging 202 millirads (range 60 to 282). Thus the total radiation resulting from Fe^{59} was, on the average, less than that due to natural sources.

The first transfusion given in any pregnancy was at 230 days antepartum; the last was usually given within 10 days before delivery. The total of 400 to 700 ml. whole blood, transfused in units of 100 ml. over a period of four to seven months, was considered insufficient to distort the normal hematology of pregnancy. Seven to 15 ml. of maternal venous blood and cord blood were obtained at delivery, and similar amounts of the infants' venous blood obtained at varying ages thereafter. After heparinization, aliquots of each sample were used for R.B.C., W.B.C. and Hgb. determinations, total iron analyses and Fe^{59} measurements. All procedures followed standard practices. The Fe and Fe^{59} measurements were carried out by the methods described by Peacock, Evans, Irvine, Good, Kip, Weiss, and Gibson (5).

RESULTS

By the breakdown of the donor cells transfused during pregnancy, radioactive iron was released

[1] Some of these data were reported at the Annual Meeting, American Pediatric Society, French Lick, Indiana, May 6, 1950.

[2] Supported in part by research grants from the Committee on Growth, American Cancer Society, to the Department of Pediatrics, Harvard Medical School, and from the National Institutes of Health to Harvard Medical School and the Massachusetts Institute of Technology; also by the joint program of the Office of Naval Research and the U. S. Atomic Energy Commission (H.M.S. and M.I.T.)

[3] The newly recommended, but still unofficial, unit of energy absorption is the rad = 100 ergs per gm. One rad = 1.20 rep_{as} = 1.07 rep_{ss}.

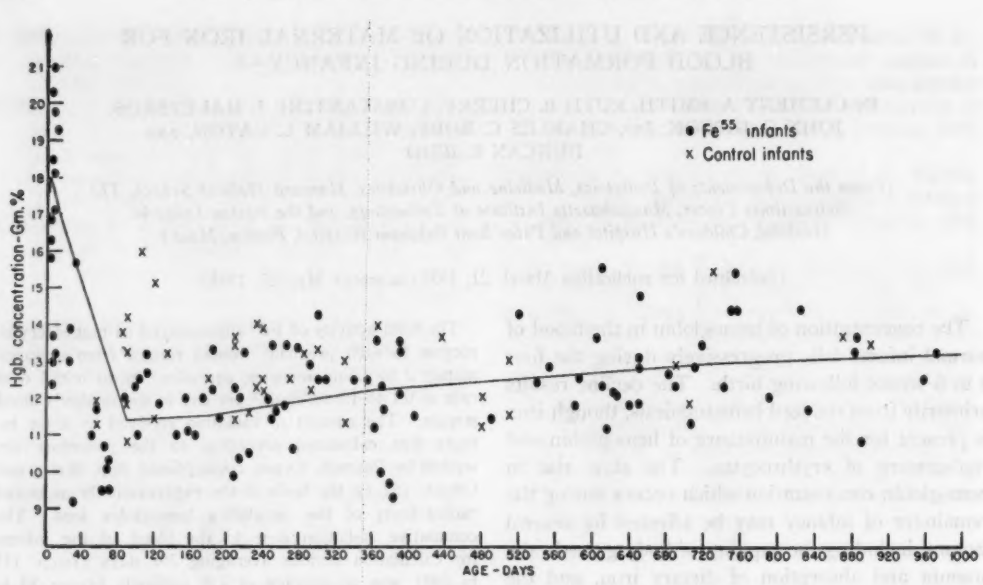

FIG. 1. HEMOGLOBIN CONCENTRATIONS OF INFANTS STUDIED COMPARED TO CONTROLS OF SIMILAR AGE

Controls consist of a group of normal infants, represented by X's (39 measurements on 33 infants), whose hemoglobins were measured concurrently with those of the infants whose blood contained Fe^{55}. Line represents course of average normals from Diamond, Smith, and Vaughan (4).

into the general pool supplying the mother and fetus. Therefore, the blood of each infant at birth contained a measurable ratio of radioactive iron to packed red cells, from which the ratio of radioactive iron to hemoglobin and to hemoglobin iron was calculated. The latter ratio, referred to as specific activity, has been normalized to equal 100 per cent for each infant's cord blood sample. Postnatal changes in the specific activities are shown in Table I (Col. 10) and Figure 2. If each infant's hemoglobin had continued to be manufactured entirely from iron obtained transplacentally, all lines of the figure would have been horizontal at the 100 per cent level. The degree of downward trend with time is a measure of post-natally acquired iron used in blood formation. Thus, a specific activity of 75 per cent would indicate utilization of dietary iron for 100–75 = 25 per cent of the infant's total circulating hemoglobin.[4]

The slight rise shown by the specific activities of blood samples obtained from several infants shortly after birth is presumably related to the timing of maternal iron administration. Since several transfusions of donor cells were given during the 11 pregnancies in which maternal hematology was under study, fetal hematopoiesis utilized iron with an increasing component of Fe^{55}

[4] Since the specific activity of only the circulating hemoglobin was determined and since several pools or compartments are involved in the dynamics of iron metabolism, constancy of circulating hemoglobin iron specific activity could have an alternate explanation. The alternative explanation would rest upon the assumption that the fetus and infant had a compartment of stored iron with specific activity much higher than that of the circulating hemoglobin. Were this the case, dietary iron might replace original hemoglobin iron in the blood and the specific activity still be maintained constant by additional incorporation into hemoglobin of small, essentially negligible, amounts of iron from this special store. The fetuses, however, were supplied with iron which constantly contained Fe^{55}. In 11 instances, Fe^{55} was continuously present in the maternal blood during the latter two-thirds of gestation, although in slowly increasing amounts. In the other 5, Fe^{55} was present in the maternal blood in a constant amount throughout the entire gestation period. In either case it would be expected that the specific activities of all the iron compartments in the body of any infant would be very nearly equal to each other at birth. The above assumption, then, would be unlikely and the alternative explanation improbable.

TABLE I

(1) Infant and Specimen number	(2) Gesta. age, Sex, etc.	(3) Age (*days*)	(4) Wt. (Kg.)	(5) Length (*cm.*)	(6) Hgb. (*Gm.* %)	(7) R.B.C. ($\times 10^6$)	(8) Hct. (%)	(9)* Cpm/ml. packed R.B.C.	(10)† "Normalized" specific activity (%)	(11)‡ Blood vol. (*ml.*) (calc.)	(12) (13) (14)§ Circulating Hgb. (*gm.-calc.*) Total	From transpl. Fe	From dietary Fe
5GB-A	~36 wks.	Birth	2.53	49	20‖	6.2	54.3	185	100	208	41.6	41.6	0
B	♂	7	2.44	49	19.8	5.5	50.0	179	90	192	38.0	34.2	3.8
C		65	5.45	55	10.7	3.3	31.7	143	84	374	40.0	33.6	6,4
D		209	7.29	62	12.3	3.9	41.4	71	47	498	61.3	28.8	32.5
E		309	8.37	67	12.5	6.0	38.3	82	50	575	71.9	36.0	35.9
F		355	8.4	69	12.5	5.9	37.5	—	—	589	73.6	—	—
G		616	9.7	77	11.2	4.2	35.7	62	39	701	78.5	30.6	47.9
H		712	10.0	79	12.9	4.4	36.8	52	30	730	94.2	28.3	65.9
I		884	11.1	83	13.2	4.8	39.7	37	22	806	106.4	23.4	83.0
J		973	11.7	84	13.2	4.1	38.0	31.4	18	833	110.0	19.8	90.2
5GB2-A	~38 wks.	Birth	2.95	47	15.0	4.4	46.0	93	100	232	34.8	34.8	0
B	Sister of	2	2.72	47	16.9	4.3	46.5	106	102	198	33.5	34.2	−0.7
C	5GB	9	2.89	47	13.2	3.5	39.6	92	97	207	27.3	26.5	0.8
D	♀	104	5.56	58	12.5	4.3	37.2	97	101	397	49.6	50.1	−0.5
E		193	6.9	60	11.5	4.2	33.4	74	75	461	53.0	39.8	13.2
6GB-A	~38 wks.	Birth	3.8	56	20‖	—	65.7	62.8	100	332	66.4	66.4	0
C	♂	12	3.77	56	19.3	5.0	64.0	68.2	110	304	58.7	64.6	−5.9
D		75	6.81	62	10.3	3.3	30.9	70.3	103	479	49.3	50.8	−1.5
E		245	10.5	74	12.0	4.0	40.6	45.2	74	709	85.1	63.0	22.1
F		392	12.1	79	13.5	5.1	41.6	41.6	62	802	108.3	67.1	41.2
G		644	14.2	88	12.9	4.4	37.1	35	49	955	123.2	60.4	62.8
H		719	14.5	91	13.4	4.9	37.5	33	45	994	133.2	59.9	73.3
I		959	16.0	95	12.5	4.9	35.4	24	33	1,079	134.9	44.5	90.4
6GB2-A	~40 wks.	Birth	3.70	56	18.2	4.7	56.1	134	100	308	56.1	56.1	0
B	Brother	6	3.38	56	18.5	5.1	53.3	145	101	283	52.4	52.9	−0.5
C	of 6GB	57	4.99	58	11.7	3.6	30.1	157	98	374	43.8	42.9	0.9
D	♂	112	6.71	63	12.7	3.7	32.0	162	99	479	60.8	60.2	0.6
E		490	12.3	76	11.5	4.7	36.0	70	53	787	90.5	48.9	41.6
7GB-A	~41 wks.	Birth	3.24	50	14.6	4.5	40.4	93.5	100	248	36.2	36.2	0
B	♀	2	2.95	50	14.3	4.5	47.9	94.8	123	223	31.9	39.2	−7.3
C		6	3.12	50	14.5	—	49.3	85.8	113	233	33.8	38.2	−4.4
D		25	3.52	54	13.0	4.1	39.8	88.8	105	277	36.0	37.8	−1.8
E		213	7.16	66	12.0	4.0	41.6	81.0	109	521	62.5	68.1	−5.6
F		374	8.6	74	11.7	4.9	37.6	50.0	62	639	74.8	47.1	27.7
G		612	10.6	80	12.3	4.2	33.8	26.7	28	761	93.6	26.2	67.4
H		793	11.4	87	12.5	4.8	36.4	33.6	38	843	105.4	40.1	65.3
8GB-A	~38 wks.	Birth	3.34	52	16.5‖	—	44.6	277	100	261	43.1	43.1	0
B	♀	7	3.04	52	17.1	5.1	49.7	321	124	242	41.4	51.3	−9.9
C		36	4.4	57	—	—	33.6	332	—	341	—	—	—
E		265	8.8	74	13.4	4.5	43.4	170	73	643	86.2	62.9	23.3
F		367	9.5	78	13.5	4.7	52.3	128	66	703	94.9	62.6	32.3
G		650	11.9	87	13.1	4.6	40.7	122	51	868	113.7	58.0	55.7
H		825	13.4	93	14.4	4.8	39.1	116	42	967	139.2	58.5	80.7
9GB-A	~40 wks.	Birth	3.02	50	19‖	—	56.9	537	100	252	47.9	47.9	0
B	♀	2	2.67	50	18.8	5.6	51.4	572	97	209	39.3	38.1	1.2
C		6	2.87	50	16.7	5.0	46.6	510	89	221	36.9	32.8	4.1
D		197	8.2	66	12.0	3.9	42.2	410	90	560	67.2	60.5	6.7
E		366	10.2	74	12.5	4.8	38.6	364	70	692	86.5	60.6	25.9
F		623	11.8	80	11.8	4.3	33.6	324	57	800	94.4	53.8	40.6
G		744	13.0	85	12.3	4.6	34.1	241	42	885	108.9	45.7	63.2

* Observed cpm/ml. corrected for decay.

† $\frac{\text{Specific activity at } t = t}{\text{Specific activity at } t = 0 \text{ (birth)}}$.

‡ Blood volumes calculated from: A. Surface area by Du Bois and Du Bois (6) formula: S.A. = $Wt.^{0.425} \times L.^{0.72} \times 71.84$; S.A. in sq. M., Wt. in Kg., L. in cm. B. Regression equation for volumes at birth calculated from data of Mollison, Veall, and Cutbush (7) on 28 normal infants: $y = 58.9 + 0.456X$; y = blood vol. in ml./Kg., X = hct. in Col. 8 (this table) × 0.95 (as correction for trapped plasma to conform with hcts. used by Mollison *et al.*). C. Regression equation for volumes after birth derived from data of Russell (8) on 40 normal infants and of Karlberg and Lind (9) on 64 normal infants: $y = 1937X - 149$; y = blood vol. in ml., X = S.A. in sq. M.

§ For negative values in this column see comment in legend of Figure 4B.

‖ Measurement not made; birth hgb. assumed from birth hct. and values from next blood sample.

TABLE I—*Continued*

(1) Infant and Specimen number	(2) Gesta. age. Sex, etc.	(3) Age (*days*)	(4) Wt. (*Kg.*)	(5) Length (*cm.*)	(6) Hgb. (*Gm.* %)	(7) R.B.C. (X10⁶)	(8) Hct. (%)	(9)* Cpm/ml. packed R.B.C.	(10)† "Normalized" specific activity (%)	(11)‡ Blood vol. (*ml.*) (calc.)	(12) Circulating Hgb. (*gm.-calc.*) Total	(13) From transpl. Fe	(14)§ From dietary Fe
11GB-A	~38 wks.	Birth	2.77	47	16.8	5.1	48	42.3	100	221	37.1	37.1	0
B	♂	7	2.64	47	13.0	4.0	35.5	44.5	100	194	25.2	25.2	0
C		64	5.27	57	9.5	3.3	29.6	41.5	107	380	36.1	38.6	−2.5
D		216	9.7	72	13.2	4.2	41.2	27.0	70	663	87.5	60.4	27.1
12GB-A	~40 wks.	Birth	3.61	49	20.5	6.2	67.4	267	100	318	65.2	65.2	0
B	♂	2	3.26	49	15.8	5.0	49.5	362	129	236	37.3	48.1	−10.8
C		6	3.44	49	13.7	4.2	51.4	296	126	246	33.7	42.5	−8.8
D		127	7.21	65	11.9	3.8	38.2	232	85	515	61.3	52.1	9.2
E		225	8.4	70	13.3	5.8	39.0	—	—	601	79.9	—	—
F		372	9.8	74	10.0	4.8	36.9	260	109	676	67.6	73.7	−6.1
G		541	10.9	80	12.4	5.2	38.6	198	70	771	95.6	66.9	28.7
H		757	11.8	87	14.4	4.7	39.4	147	46	864	124.4	57.2	67.2
12GB2-A	~40 wks.	Birth	3.66	53	16.9	4.6	56.0	143	100	305	51.5	51.5	0
B	Sister of 12GB ♀	7	3.46	53	17.8	5.1	57.6	146	100	269	47.9	47.9	0
13GB-A	~39 wks.	Birth	3.2	45	20.3	5.0	58.4	156	100	269	54.6	54.6	0
B	♀	2	2.86	45	12.9	4.2	40.4	160	111	192	24.8	27.5	−2.7
C		6	2.92	45	12.4	3.9	35.5	189	120	196	24.3	29.2	−4.9
D		70	5.1	59	9.5	3.6	27.8	177	115	386	36.7	42.2	−5.5
E		153	7.4	66	12.0	4.0	38.3	—	—	531	63.7	—	—
F		373	10.0	78	12.3	4.6	35.3	123	79	719	88.4	69.8	18.6
G		526	12.1	83	14.2	4.4	38.8	76	46	837	118.9	54.7	64.2
15GB-A	~42 wks.	Birth	3.77	55	17.1	5.1	57.9	165	100	317	54.2	54.2	0
B	♂	5	3.63	55	20.3	6.0	62.1	186	102	291	59.1	60.3	−1.2
C		31	4.37	57	15.7	5.1	47.6	194	105	339	53.2	55.9	−2.7
D		85	5.9	62	12.0	3.5	35.8	192	103	438	52.6	53.7	−1.1
E		250	8.9	71	13.4	5.4	46.5	118	73	626	83.9	61.2	22.7
F		389	10.6	77	12.1	5.7	36.6	118	64	736	89.1	57.0	32.1
16GB-A	~40 wks.	Birth	3.04	48	18.8	5.7	62.5	90¶	100	261	49.1	49.1	0
B	♂	7	2.96	48	18.1	5.1	56.3	93	97	217	39.3	38.1	1.2
C		50	4.43	56	11.0	3.6	29.9	102	93	335	36.9	33.9	3.0
D		102	5.87	64	11.0	3.4	32.2	109	106	450	49.5	52.5	−3.0
E		185	7.48	69	12.2	4.0	36.9	86	87	556	67.8	58.3	9.5
F		382	9.8	76	9.7	3.4	30	66	68	699	67.8	46.1	21.7
G		576	10.8	81	11.8	3.8	32.6	42	39	777	91.7	35.8	55.9
H		718	11.6	86	11.3	4.0	33.7	35	35	847	95.7	33.5	62.2
I		891	12.5	90	10.8	4.0	27.7	28.7	25	916	98.9	23.7	75.2
J		961	12.5	92	10.8	3.5	32.4	32.5	33	934	100.9	33.3	67.6
16GB2-A	~40 wks.	Birth	3.01	52	21.6	5.2	64.5	309	100	262	56.6	56.6	0
B	Brother of 16GB and 16GB3 ♂	7	3.09	52	21.0	5.4	55.7	333	96	246	51.7	49.6	2.1
C		27	3.57	56	13.9	4.8	41.0	326	104	295	41.0	42.6	−1.6
D		210	7.5	70	10.4	4.5	33.8	230	81	562	58.4	47.3	11.1
E		293	8.45	75	10.4	4.3	29.7	206	64	641	66.7	42.7	24.0
F		383	9.2	78	9.3	4.6	33.4	216	84	694	64.5	54.2	10.3
G		453	9.5	83	9.6	5.2	32.9	186	69	742	71.2	49.1	22.1
16GB3-A	~40 wks.	Birth	3.4	50	19.3	4.4	60.2	185	100	289	55.8	55.8	0
B	Sister of 16GB and 16GB2 ♀	5	3.18	50	19.8	4.6	57.4	203	102	236	46.7	47.6	−0.9
C		48	4.51	58	10.5	3.3	30.6	218	110	353	37.1	40.8	−3.7
17GB-A	~42 wks.	Birth	4.39	56	15.8	5.1	49	82	100	352	55.6	55.6	0
B	♂	2	3.77	56	16.3	5.1	49.4	86	103	304	49.6	51.1	−1.5
C		7	4.23	56	13.7	5.0	46.4	85	113	328	44.9	51.2	−6.3
D		363	12.2	80	11.2	4.4	36.1	62	79	818	91.6	72.4	19.2

¶ Sample lost in laboratory; cpm/ml. assumed on basis of 7-day sample and trend in counts for other infants.

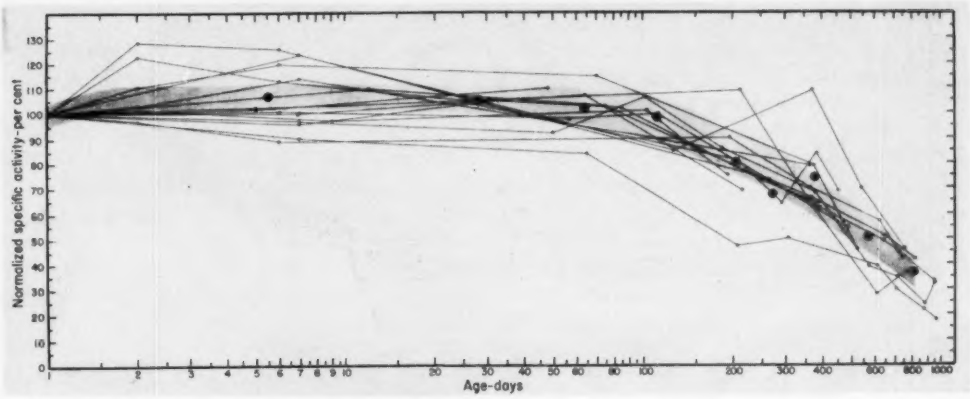

FIG. 2. NORMALIZED SPECIFIC ACTIVITIES OF 16 INFANTS UP TO 32 MONTHS AFTER BIRTH

The lines connect consecutive data for each individual infant. The black dots (unconnected) indicate average values for the means of the following age groups:

1. Birth
2. 2–12 days
3. 13–31 days
4. 32–100 days
5. 101–150 days
6. 151–216 days
7. 217–309 days
8. 310–400 days
9. 401–675 days
10. 676–973 days

The shaded section indicates the area bounded by 2 standard errors above and below the best curve through the average values.

The average normalized specific activities above 100 per cent are interpreted as resulting from circumstances of maternal Fe^{55} administration (see text).

as birth approached. As a result, hemoglobin in fetal erythrocytes made shortly before birth must have contained more Fe^{55} than hemoglobin in cells of earlier manufacture. Survival of these younger cells after degeneration of the older ones would produce the effect observed. Blood from the five infants born after the later pregnancies during which no Fe^{55} was introduced showed little or no specific activity rise in the early post-natal period.

The relatively constant level maintained by the specific activities plotted in Figure 2 until approximately 100 days after birth indicates no appreciable use of food iron for hemoglobin formation during this time. Thereafter, dietary iron appears in the circulating hemoglobin in progressively increasing amounts. At 800 days (26 months) for example, approximately 20 to 50 per cent of the hemoglobin is still formed from transplacental iron, the remaining 50 to 80 per cent from dietary iron.

A statement of the actual amounts of transplacental and dietary iron in the total circulating hemoglobin requires knowledge of blood volume. This could not be directly obtained but an approximate value was calculated from physical measurements of each infant at each sampling. The formulae used for derivation of blood volumes according to the data of other investigators (7–9) are described in the note under Table I, and the calculated blood volumes are shown in Column 11 of that table.

The total amounts of circulating hemoglobin, computed from calculated blood volumes and measured hemoglobin concentrations, are listed in Column 12 of Table I and plotted in Figure 3. The mean total circulating hemoglobin at 2 to 3 months of age was approximately equal to the mean at birth; the mean at 1½ to 2 years of age was twice that at birth.

The amounts of hemoglobin iron of transplacental and dietary origin, calculated from specific activity measurements, are listed in Table I and shown graphically in Figures 4A and 4B. Figure 4A indicates that the mean amount of hemoglobin made from transplacental iron remains essentially constant throughout the period of these investigations. However, the elevation of the mean by some 20 per cent above the birth level

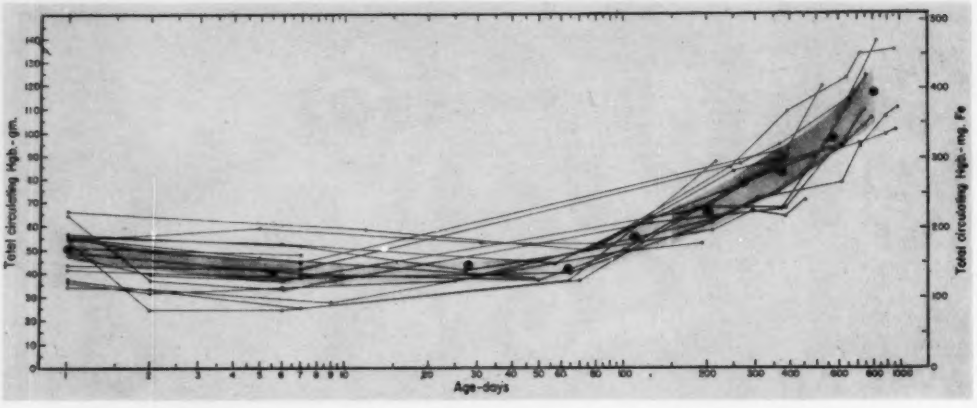

FIG. 3. TOTAL CIRCULATING HEMOGLOBIN OF 16 INFANTS UP TO 32 MONTHS AFTER BIRTH
(For details of plotting see Figure 2.)

at approximately 200 to 400 days suggests hemoglobin formation from transplacental iron in addition to that which was in hemoglobin at birth. In the following discussion, such iron will be referred to as "stored" transplacental iron even though hemoglobin at birth is also in a sense a storage depot. One cannot assume, in view of the wide statistical confidence limits of the data, that stored transplacental iron regularly represents 20 per cent more than that in hemoglobin at birth;

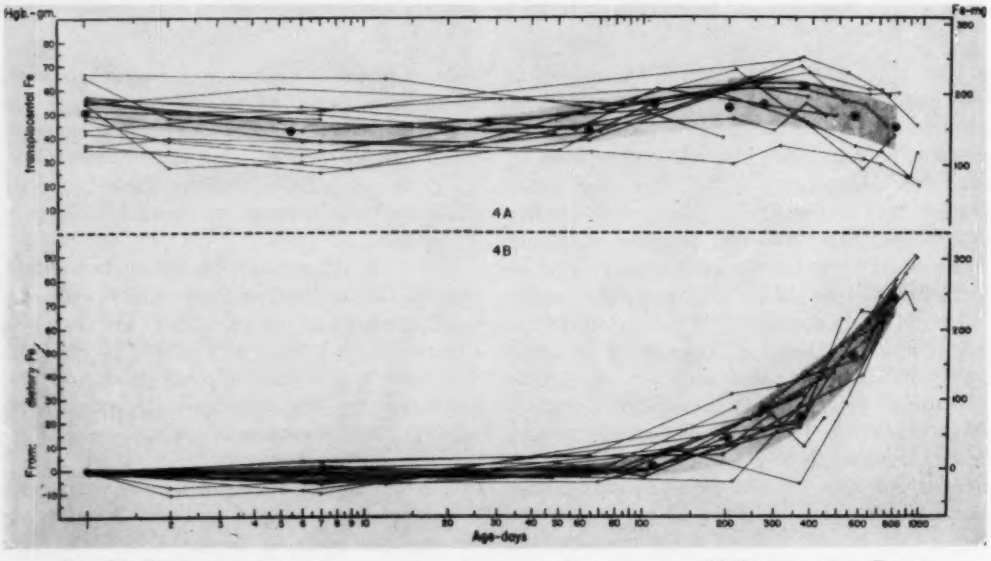

FIG. 4A. HEMOGLOBIN FROM TRANSPLACENTAL IRON OF 16 INFANTS UP TO 32 MONTHS AFTER BIRTH

FIG. 4B. HEMOGLOBIN FROM DIETARY IRON OF 16 INFANTS UP TO 32 MONTHS AFTER BIRTH

Negative values for individual and average hemoglobins from dietary iron may arise partly from statistical considerations and partly from the fact that some of the normalized specific activities of Figure 2 are greater than 100 per cent.

(For details of plotting see Figure 2.)

nevertheless, the findings suggest that at least some iron is available to the infant from non-hemoglobin stores.

Similarly, the data as a whole do not allow the conclusion that the terminal downward trend of the mean in Figure 4A definitely indicates a loss of transplacental iron from the blood, even though such a loss appears to have occurred in individual infants, as will be discussed below. The decline of specific activities shown in Figure 2 results primarily from dilution with dietary iron. Figure 4B indicates the average amount of hemoglobin made from iron obtained from the diet: essentially none before the 4th month,[5] about 10 grams by 6 months, 22 grams by 1 year, and 65 grams by 2 years.

DISCUSSION

Direct measurement of iron intake and loss was not attempted in these studies. Relatively little information has been found in the literature regarding the amount of iron normally absorbed and excreted by infants, particularly during the first few months after birth. Oettinger, Mills, and Hahn (10) have demonstrated that tagged iron administered by stomach tube is absorbed by term and premature infants at a few days of age. At least under the experimental conditions of that study, the rate of incorporation of iron into hemoglobin is extremely small (0.01 μg. Fe per day) compared to total circulating hemoglobin (150 mg. Fe). Incorporation of 0.01 μg. Fe per day would not have been detected by the methods of the present study. Oettinger, Mills, and Hahn (10) gave no data regarding the excretion of the test iron. Balance studies by other workers (1) indicate that infants of less than 3 months are in net iron equilibrium on a daily intake of about 0.15 mg. per Kg., and in negative balance on smaller iron intakes. Such figures do not differentiate between iron which fails to be absorbed and that which is excreted from endogenous sources into the bowel, except as excretion may be inferred from the negative balances when the diet contains less than 0.15 mg. per Kg. If the infant's endogenous iron loss is actually 0.15 mg. per Kg. per day, that amount would be disproportionately large compared to the total daily iron loss of 1.0 mg. usually assumed for the 70 Kg. adult.

Thus while it has been demonstrated by others that the infant may both absorb and excrete iron, the amounts have not been accurately determined. The present investigation suggests absorption and loss of much less iron than is implied by available balance data. In the infants of this study, as a group, there was no indication of either absorption or loss during a period extending into the fourth month after birth. The decline of hematopoiesis (11) and reduction in mean red blood cell size and mean corpuscular hemoglobin (12) normally occurring in this age period, together with dilution by body growth and blood volume expansion, produce the fall in hemoglobin concentration apparent in Figure 1. The total amounts of hemoglobin of the infants studied are shown by the mean in Figure 3 to have remained fairly steady until a rising trend appeared at about 60 days. Thus, the amount of hemoglobin began to increase 1 to 2 months before there was evidence of any dietary iron in the blood (Figure 4B). Transplacental iron was apparently sufficient for the beginning expansion of circulating hemoglobin.

Between the third or fourth month and the end of the second year of age, the concentration of hemoglobin increased only slightly, while the total amount of hemoglobin was doubled, primarily by addition of that made from dietary iron. The data presented in Figure 4A indicate that little of the original hemoglobin iron left the blood during this period, whereas some transplacental iron from stores may have participated in the increase in hemoglobin.

The amounts of transplacental iron apparently emerging from "stores" varied in the individual infants. It might be expected that the total amount of iron in hemoglobin at birth would parallel the amount stored elsewhere and that an infant born with a relatively low total circulating hemoglobin would have comparatively little stored iron for later blood formation. To test this possibility, the largest addition of hemoglobin from stored iron for each of the 14 infants studied for more than 100 days was plotted against the total hemoglobin of that infant at birth (Figure 5).

[5] Assuming constant daily incorporation of dietary iron into hemoglobin resulting in a significant decrease in the specific activity at 4 months, the rate of incorporation would have to be at least ~ 50 μg. Fe per infant per day to be detectable at this time.

Except for one instance (Infant 5GB), the data suggest that infants with low total hemoglobin at birth seemed to have relatively large amounts of stored iron available for later hemoglobin manufacture.

It cannot be argued that those infants born with much circulating hemoglobin were necessarily lacking in iron stored elsewhere, since they may not have been required to draw upon such iron later. Nevertheless, it is apparent that the full-term infant's iron resources cannot be predicted from his hemoglobin concentration and body size at birth. Some prediction of low fetal storage can be made when the maternal hemoglobin concentration in late pregnancy is pathologically low, as shown by the work of Strauss (13). But Woodruff and Bridgeforth (14) found no such correlation when the hemoglobin concentration of the mother's blood remains above 9 grams per cent. In the present investigation, the hemoglobin of one mother fell to 10.2 grams per cent; all others had more than 11 grams per cent hemoglobin during the last trimester of their pregnancies.

Data from infants followed sufficiently long indicate that dietary iron not only continues to be added to transplacental iron but gradually begins to replace it in hemoglobin formation during the third year, as is suggested by the late downward trend of the mean in Figure 4A.

The preceding discussion applies to the general pattern of iron utilization in the infants as a group. Variations shown by certain individuals deserve brief presentation. Perhaps the most interesting was Infant 5GB. While not premature by the usual standards of weight, this infant was born one month before expected date of confinement. The evidence obtained from ten blood samples over a period of 973 days is shown in Figure 6. The infant, a Negro male, gained weight slowly after two months of rapid growth. In spite of two rather severe infections he had no definite anemia, though medicinal iron (ferrous sulfate, 240 mg. per day) was prescribed for 6 weeks in his second year when his hemoglobin was found to be 11.2 grams per cent. His mother's dietary intake of iron during pregnancy was rated as very poor; his own diet was relatively high in iron during the first six months and then somewhat lower but still adequate.

Because of his small size this infant's total circulating hemoglobin was only 42 grams at birth. As Figure 6 shows, dietary iron appeared in his hemoglobin by 65 days after birth. This process continued so consistently that by 2 years only 30 per cent of the iron in his hemoglobin was of trans-

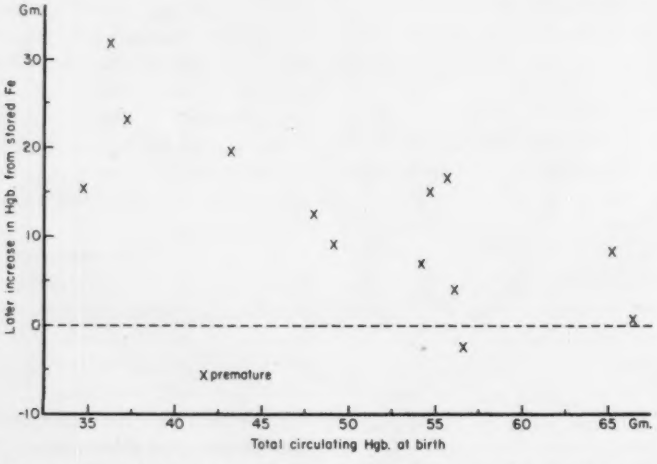

FIG. 5. RELATION BETWEEN MAXIMUM EMERGENCE OF TRANSPLACENTAL IRON AFTER BIRTH AND TOTAL CIRCULATING HEMOGLOBIN AT BIRTH

(Fourteen infants; omitting Infants 12GB2 and 16GB3, studied less than 100 days.)

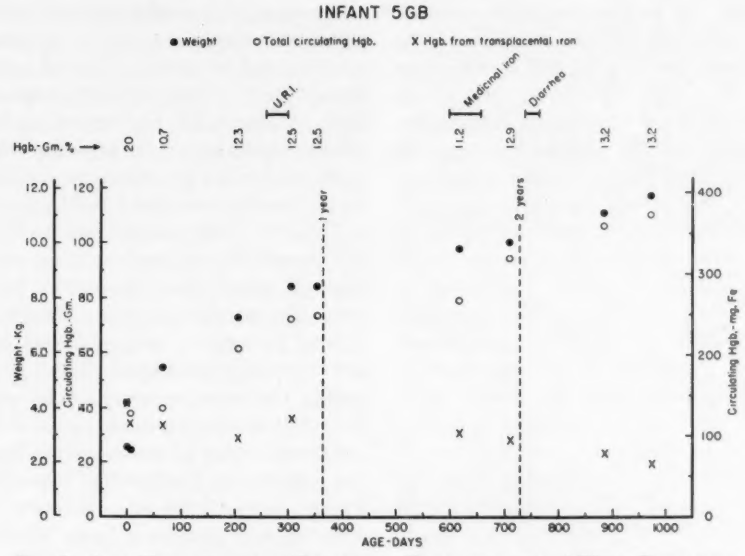

FIG. 6. IRON UTILIZATION FOR HEMOGLOBIN FORMATION IN A 4-WEEK PREMATURE, 2.53 KG. INFANT; BIRTH TO 32 MONTHS OF AGE

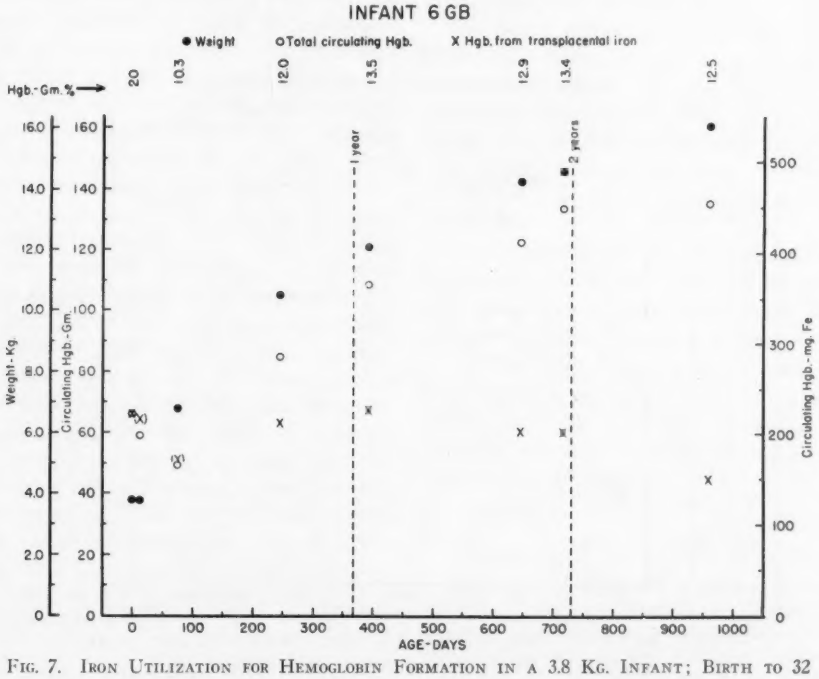

FIG. 7. IRON UTILIZATION FOR HEMOGLOBIN FORMATION IN A 3.8 KG. INFANT; BIRTH TO 32 MONTHS OF AGE

Values of hemoglobin from transplacental iron greater than the total circulating hemoglobin (indicated by parenthetic symbols) arise from normalized specific activities above 100 per cent, discussed in text.

placental origin. At no time was there evidence of stored iron appearing in blood formation. The provision of medicinal iron at 600 to 640 days was followed by a slight increase in hemoglobin concentration and in total circulating hemoglobin. The data suggest that the increased hemoglobin was not manufactured from transplacental iron.

In summary, this infant, born prematurely to a poorly nourished mother, was outstanding among the group by virtue of his low total hemoglobin at birth and the consistently small contribution of transplacental iron to later hemoglobin formation. Both of these features might well be consequences of prematurity. About 70 mg. of iron originally present had disappeared from this infant's blood at 32 months. Although not excessive in amount when compared to the trend of mean values, 70 mg. of iron represented an inordinately large proportion of that acquired by this infant during fetal life. The data further suggest that iron added to this infant's oral intake was utilized for hemoglobin formation.

Infant 6GB (Figure 7) affords an interesting comparison. His mother had been well-nourished before and during pregnancy. His own diet was excellent and he remained free of infections. Although born 13 days before the expected date, his birth weight of 3.8 Kg. argues against any significant prematurity. He gained about 30 per cent more weight than the infant just considered during the 32 months over which both were followed.

The iron metabolism of this infant and that of the premature one just discussed are similar in many respects. Their hemoglobin concentrations were similar at birth and thereafter. Neither showed evidence of any significant utilization of stored transplacental iron. Both formed approximately the same amounts of hemoglobin from dietary iron at comparable ages. Finally, about the same amount of transplacental iron (70 mg.) had apparently disappeared from the blood of both at 32 months of age. However, Infant 6GB was not only larger at birth but gained more weight thereafter. As a result, he consistently maintained a higher total of circulating hemoglobin, a relatively larger proportion of which was

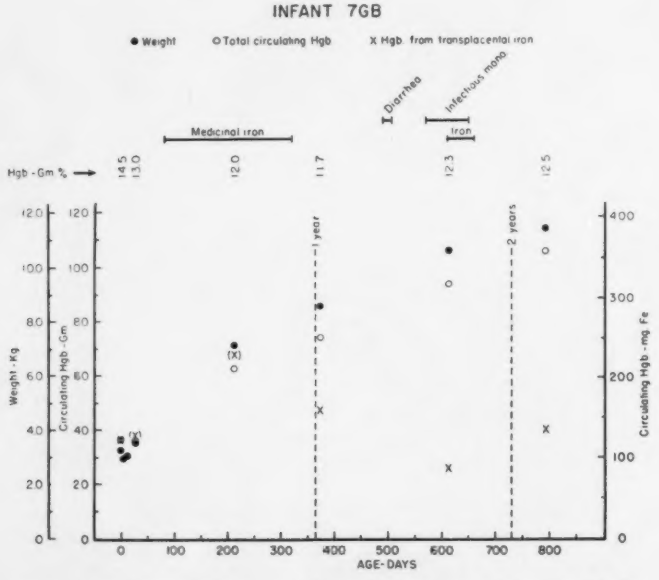

FIG. 8. IRON UTILIZATION IN A 3.24 KG. INFANT BORN WITH LOW TOTAL CIRCULATING HEMOGLOBIN; BIRTH TO 26 MONTHS OF AGE

Data show emergence of transplacental iron from stores during first 200 days, and suggest similar emergence between 600 and 800 days. For comment on parenthetic symbols, see legend of Figure 7.

made from the transplacental iron present in his hemoglobin at birth. It is perhaps unfortunate that this infant had so good a diet. Otherwise his resources of stored transplacental iron might have become apparent. As they stand, his data indicate the large contribution which may be made by the hemoglobin at birth to iron needs throughout infancy.

In contrast to the two infants just discussed, the data from Infant 7GB (Figure 8) indicated the emergence of considerable transplacental iron from stores. This infant was born 9 days after expected confinement to a mother whose diet had been good. Birth weight was 3.24 Kg. The hemoglobin concentration of 14.6 gm. per cent in cord blood was low, without apparent reason from the obstetrical history or studies made during this mother's pregnancy (3). The similarity of hemoglobin concentrations in samples taken at 2 and 6 days after birth substantiates the measurement from cord blood.

The infant was fed from the breast for 120 days, and thereafter artificially but on an adequate diet. For unknown reasons, the family physician prescribed ferrous sulfate in dosages from 40 to 160 mg. daily for most of the first year. The data of Figure 8 suggest that none of this iron was used for hemoglobin during the first 7 months, whereas about 100 mg. of transplacental iron, which could not have been in the blood at birth, apparently entered the circulating hemoglobin by 200 days. Similar emergence of iron from stores in other infants born with low concentrations or total amounts of hemoglobin has been commented upon above. From 200 to 600 days, transplacental iron in hemoglobin decreased to below the birth level. At some time between 600 and 800 days after birth, and following a moderate attack of infectious mononucleosis, a second emergence of transplacental iron is suggested by the data. This infant's blood showed approximately the same calculated amount of transplacental iron at 26 months of age as was in her hemoglobin at birth. It is difficult to relate the two periods of medicinal iron administration to the appearance of iron from storage in this child's course.

SUMMARY

Sixteen infants of mothers who had received transfusions of red blood cells containing Fe^{55} were studied for periods up to 32 months of age. Measurements of R.B.C., Hgb., Hct., total iron and Fe^{55} of erythrocytes, were made from umbilical cord blood and from venous blood obtained at intervals during the study period.

Results from the 16 infants as a group indicated very little or no utilization of dietary iron for hemoglobin until 3 to 4 months after birth. At 1 year, iron of transplacental origin constituted ~ 70 per cent of total hemoglobin iron; at 2 years, ~ 40 per cent. In absolute terms these values correspond to ~ 170 mg. transplacental iron at birth, ~ 200 mg. at 1 year, and ~ 160 mg. at 2 years in the total circulating hemoglobin. At 2 years, over 90 per cent of transplacental iron was still utilized for hemoglobin.

A 20 per cent rise in transplacental iron at 200 to 400 days over that present in hemoglobin at birth suggested utilization of iron stored elsewhere during fetal life. Infants with relatively low total circulating hemoglobin at birth tended to show relatively large amounts of such stored iron in later hemoglobin manufacture.

Individual infants deviated from the pattern described for the group. In particular, a premature infant of 36 weeks' gestation had low total circulating hemoglobin at birth, no evidence of any stored iron in later hemoglobin formation and proportionately high utilization of dietary iron, which first appeared in hemoglobin at 65 days after birth.

It is concluded that the hemoglobin of normal infants (A) utilizes iron obtained during fetal life throughout infancy, (B) incorporates significant amounts of dietary iron only after 3 or 4 months of age, and (C) by two years reaches a total of ~ 115 grams, composed of ~ 70 grams from dietary and ~ 45 from transplacental iron. Prematurity increases the proportionate contribution of dietary iron.

ACKNOWLEDGMENTS

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ALTERATIONS IN MUSCLE TENSION WITHOUT SIMILAR CHANGES IN ELECTRICAL ACTIVITY IN PATIENTS WITH MYASTHENIA GRAVIS [1]

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Certain discrepancies between the electrical and mechanical activity of skeletal muscle in patients with myasthenia gravis have been pointed out previously (1). However, simultaneously recorded action potentials and tension have been reported only once in patients with this disorder (2) and no quantitative analysis has been made of the relationship between electrical and mechanical activity of skeletal muscle of patients with myasthenia gravis. The results of the present study of simultaneously recorded electrical and mechanical activity of intact muscle in six patients with myasthenia gravis indicate that changes in tension reflect more accurately the degree of muscle weakness than do changes in electrical activity and changes in contractile ability of the muscle can occur, both before and after neostigmine, without similar changes detectable in neuromuscular transmission.

APPARATUS AND METHODS

The method and apparatus have been described in detail elsewhere (3). In brief, we recorded simultaneously action potentials from and isometric tension developed by the adductor pollicis muscle, which was induced to contract by supramaximal, percutaneous stimulation of the ulnar nerve at the elbow. We are cognizant of the fact that movements of the thumb are complex and therefore we might not be recording mechanical and electrical activity from the same group of muscles. However, an electrode on the belly of the adductor pollicis was certainly picking up electrical activity of muscles which are innervated by the ulnar nerve. When supramaximal stimuli were used potentials of duplicate single twitches, successive twitches with 3/sec. and successive responses with 30/sec. did not vary more than 10 per cent in 48 normal subjects. The same was true of tension. When the ulnar nerve is stimulated at the elbow, the hypothenar, forearm flexor, interosseus and palmaris brevis contract. However, if ulnar deviation, wrist flexion and finger flexion are prevented, as they were in the present study, these muscles contribute very little to the tension developed by the adductor pollicis when it is initially stretched. In fact, when these other movements occur, the bar tends to be pushed up and the recorded tension is less. Any opposition of the thumb also tends to decrease tension since an oblique rather than perpendicular force is applied to the bar. In any case with supramaximal stimuli the tension developed by successive twitches or duplicate tetanus in 48 normal subjects did not vary more than 10 per cent. In this study, we have considered only changes greater than 10 per cent to be significant (three times standard deviation of the mean of individual differences).

The ulnar nerve was stimulated either with single twitches or at the rate of 3/sec. for 2 seconds (at which rate there was no fusion of successive twitches), 2 minutes before and at intervals varying from 1 to 10 minutes after a tetanus produced by 30/sec. stimulation for 1 second. Records were taken in six patients both when they were weak but not necessarily in a basal state (at least 3 to 4 hours after their usual oral dose of neostigmine, Table 1) and again when they felt stronger (20 to 30 minutes after intramuscular injection of 1.5 mgm. neostigmine with 0.6 mgm. atropine). In addition conduction velocities along the ulnar nerve were determined in three additional patients by measuring the distance between two points of stimulation (elbow and wrist) and the difference in latencies between stimulus and artefact when the nerve was stimulated at these two points. Before tetanus the ulnar nerve was stimulated successively at elbow and wrist with single shocks to determine the difference in latency. The differences in latency of the first and fifth responses were determined when tetanus was obtained with 30/sec. for 1 second by stimulation at both wrist and elbow. By this technique one determines the conduction velocity of the fastest fibers as they conduct only from elbow to wrist.

We are fully aware of the fact that different muscle groups are variously affected in patients with myasthenia gravis and that voluntary muscle contraction does not necessarily measure the same thing that is measured when one measures tension developed with supramaximal indirect stimulation. However, the patients in this study showed a decrease in ptosis and increased strength of adduction of the thumb (as judged by their ability to

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TABLE 1

Clinical data

Patient	Sex	Age *yrs.*	Age at onset of symptoms *yrs.*	Daily oral dose of neostigmine *mgm.*	Interval between last dose of neostigmine and study *hrs.*	Calibration of EMG *mV/mm.*
D. D.	F	18	18	0	24*	.3
B. G.	F	3	3	0	24*	.4
Na-1	F	37	21	0	24*	.5
Na-2	F	44	21	30 q 4h.	4–5	.3
L. P.	M	32	25	0	24*	.3
E. R.	F	24	21	7.5 q 3h.	3–4	.3
M. W.	F	19	13	15 q 4h.	8–9	.3
M. S. W.	F	20	14	15 q 4h.	7–8	.4
M. A.	F	28	22	15 q 4h.	5–6	.4

* 1.5 mgm. neostigmine with 0.6 mgm. atropine had been given intramuscularly for diagnostic purposes 24 hours before the studies were done.

hold a sheet of paper between the thumb and first metacarpal by adduction of the thumb), 30 minutes after intramuscular neostigmine. Other pertinent clinical data are given in Table 1.

Although it is known that tension and amplitude of action potentials vary with age and initial muscle length, insufficient data are available from normal human adductor pollicis muscle to permit comparison with patients with myasthenia gravis. For this reason absolute values for amplitude of muscle action potentials are not given but they can be obtained from the calibration factors in Table 1 since the amplification system was linear and the sensitivity remained the same throughout any one study.

RESULTS

3/sec. stimulation (Table 2): Before neostigmine, there was a decrease in the tension developed by successive twitches in each patient; the tension developed by the fifth twitch was, on the average, 70 per cent that developed by the first twitch. Such a decrease in the tension of successive twitches at 3/sec. does not occur in normal subjects. There were no consistent changes in amplitudes of the accompanying action potentials, *i.e.,* there was no change in amplitude in E. R., M. W. (Figure 1) and M. S. W., and a moderate decrease in L. P.; thus per cent decrease in tension did not parallel per cent decrease in amplitude of the action potential. *After neostigmine* there was no significant change in the first twitch tension in E. R., M. W. (Figure 1) and M. S. W. and a slight increase in tension developed by the first twitch in L. P. Again there was no correlation between electrical and mechanical activity, *e.g.,* there was an increase in amplitude of the first action potential unaccompanied by an increase in tension in E. R. whereas in L. P. there was an increase in tension developed by the first twitch unaccompanied by any significant change in the action potential. The lower action potential in E. R. was not the result of a change in amplification since the calibration factor remained the same throughout this, as well as all other studies. After neostigmine, the fifth twitch developed greater tension than before in E. R., L. P. and M. S. W. so that, on the average, the fifth twitch tension was 85 per cent that of the first. There was good correlation between electrical and mechanical activity in E. R., L. P. and M. W.; however, in M. S. W., despite the greater tension developed by the fifth twitch after neostigmine, there was no increase in amplitude of the accompanying action potential. There were no significant changes in either the delay (interval between beginning of stimulus artifact and beginning of the

TABLE 2: Simultaneous tension and action potentials developed by the adductor pollicis brevis muscle with 3/sec. stimulation of the ulnar nerve at the elbow. In this and subsequent tables, changes less than 10% are not significant and the numbers 1 and 5 refer to the responses to the 1st and 5th stimulus.

Patients	Pre-neostigmine				Post-neostigmine			
	Tension gm.		Action Pot. Amplitude mm.		Tension gm.		Action Pot. Amplitude mm.	
	1	5	1	5	1	5	1	5
E.R.	580	450	19	17.5	550	470	25	26
L.P.	680	430	31	22	770	680	32	31
M.W.	760	640	32	32	775	650	33	32.5
M.S.W.	510	240	36	35	470	390	34	34
Mean	633	440	29.5	26.6	641	548	31	30.9

action potential) or latent period (interval between beginning of action potential and beginning of the rise in tension) of successive twitches at 3/sec. either before or after neostigmine.

30/sec. stimulation (Table 3): Before neostigmine. The electromyographic picture of a decrement of successive action potentials described by Harvey and Masland in 1941 (1) occurred in three patients (E. R., Figure 2, L. P. and M. S. W.) but there was no significant change in the amplitude of successive potentials in the other patient (M. W.). The conduction velocities of the ulnar nerve were normal both before and during the tetanus at a time when the action potentials had decreased in three other patients (Table 4). *After neostigmine,* there was an increase in tetanus tension in two patients (M. W. and M. S. W.) and no change in tetanus tension in the other two patients (E. R., Figure 2, and L. P.). Thus only in M. S. W. was there a correlation between the mechanical and electrical activity since only here was the increased tension accompanied by an increase in amplitude of the first potential and a decrease in decrement of successive potentials. In the other three patients there was no correlation between electrical and mechanical activity.

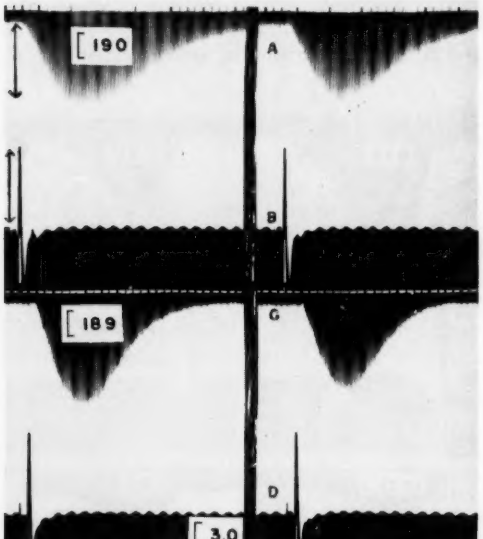

FIG. 1. PATIENT M. W. FIRST (ON READER'S LEFT) AND FIFTH (ON READER'S RIGHT) MECHANICAL (A AND C) AND ELECTRICAL (B AND D) RESPONSES OF THE ADDUCTOR POLLICIS TO 3/SEC. STIMULATION OF THE ULNAR NERVE 8 TO 9 HOURS AFTER 15 MGM. ORAL NEOSTIGMINE (A AND B) AND 20 MIN. AFTER 1.5 MGM. INTRAMUSCULAR NEOSTIGMINE (C AND D)

In this and all subsequent figures, lines A and C record tension, maximum developed tension being measured as indicated by the upper arrow; lines B and D record electrical activity, the amplitude of the negative phase being measured as indicated by the lower arrow; the small upward deflection preceding the negative phase of the action potential when present is the stimulus artifact; and each of the widest time markers indicates 0.03 sec. In this figure calibration of tension is in terms of gm./extremes of the bracket and of electrical activity in terms of mV/extremes of the bracket.

TABLE 3: 30/sec. stimulation before and 20-30 minutes after intramuscular neostigmine.

	Pre-Neostigmine			Post-Neostigmine		
Patient	Tension gm.	Action Pot. amplitude mm.		Tension gm.	Action Pot. amplitude mm.	
		1	5		1	5
E.R.	3000	22	15	3150	26	30
L.P.	3500	30	16	3200	29	25
M.W.	2950	33	32	3950	29	26
M.S.W.	3460	35	20	4200	43	37
Mean	3228	30	20.9	3625	31.8	29.5

TABLE 4: Conduction velocity of ulnar nerve (M/sec.). 1 and 30 are the responses to the 1st and 30th stimuli: Tetanus produced by 25/sec. stimulation for 1.2 sec.

Patient	Before Tetanus	During Tetanus		
	C.V.	C.V.	Act. Pot. ampl. (mm)	
			1	30
N.A.-1	55.2	55.0	16	10
B.O.	75.0	74.2	29	25
D.D.	50.7	51.1	19	9

Post-tetanic potentiation of twitch tension: Before neostigmine (Table 5): Single twitch tension, as judged by the first twitch with 3/sec. stimulation, was greater after tetanus than the corresponding twitch before tetanus. As in normal subjects (3), this potentiation of twitch tension was unaccompanied by a change in the action potential. In two other myasthenia gravis patients (N. A.-2 and M. A., Table 6) we found that al-

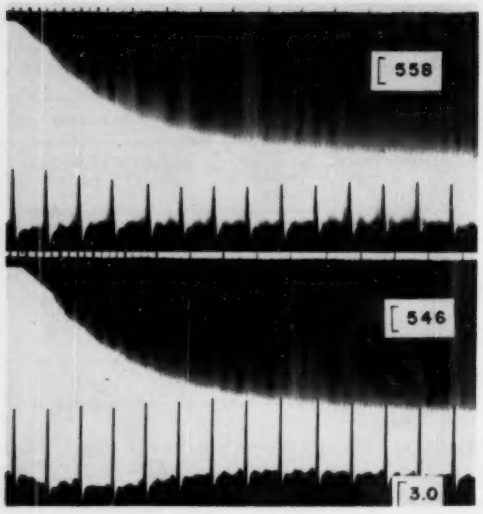

FIG. 2. PATIENT E. R. SIMULTANEOUS MECHANICAL AND ELECTRICAL RESPONSES OF THE ADDUCTOR POLLICIS TO 30/SEC. STIMULATION OF THE ULNAR NERVE 3 TO 4 HOURS AFTER 7.5 MGM. ORAL NEOSTIGMINE (UPPER TWO LINES) AND 20 TO 30 MIN. AFTER 1.5 MGM. INTRAMUSCULAR NEOSTIGMINE (LOWER 2 LINES)

In this figure, calibration of tension is in terms of gm./extremes of the bracket and of electrical activity in terms of mV/extremes of the bracket.

though the twitch tension was potentiated at 1 min. it was not potentiated 2 min. after the tetanus; this is unlike normal human subjects, in whom post-tetanic potentiation of twitch tension lasts at least 10 min. (3). After tetanus, there was a progressive decrement in tension of successive twitches with 3/sec. stimulation in all four myasthenia gravis patients; again this is unlike normal human subjects, in whom the tension of successive

TABLE 5: 3/sec. stimulation after tetanus before and 20-30 minutes after intramuscular neostigmine.

Patients	Pre-neostigmine				Post-neostigmine			
	Tension gm.		Action Pot. Amplitude mm.		Tension gm.		Action Pot. Amplitude mm.	
	1	5	1	5	1	5	1	5
E.R.	800	675	17.5	15	770	695	22.5	16.5
L.P.	800	230	33	13	1100	800	31	26
M.W.	1025	940	32.5	32	1150	975	32	32
M.S.W.	1200	190	35	27	1400	500	34	33
Mean	956	509	29.5	21.8	1105	743	29.9	26.9

TABLE 6: Single twitches before and after tetanus.

Patient	Pre-Tetanus		Post-Tetanus			
			1 minute		2 minutes	
	Tension gm.	Act. Pot. (mm)	Tension gm.	Act. Pot. (mm)	Tension gm.	Act. Pot. (mm)
N.A.-2	250	26	310	25	250	25.5
M.A.	860	35	990	36	840	35

twitches after tetanus at this stimulation rate is maintained at the potentiated single twitch level (4). This decrement in successive twitch tensions was greater after tetanus in patients L. P. and M. S. W. and about the same in patients E. R.

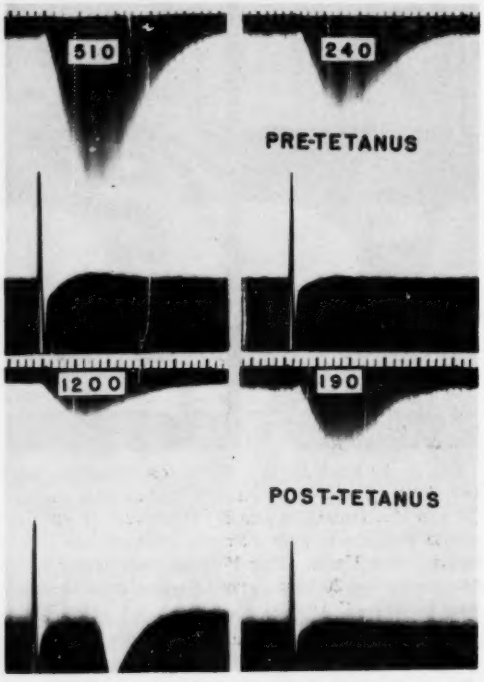

FIG. 3. PATIENT M. S. W. FIRST (ON READER'S LEFT) AND FIFTH (ON READER'S RIGHT) MECHANICAL AND ELECTRICAL RESPONSES OF ADDUCTOR POLLICIS MUSCLE TO 3/SEC. STIMULATION OF THE ULNAR NERVE BEFORE AND AFTER TETANUS OF 30/SEC. FOR 1 SEC. 7 TO 8 HOURS AFTER 15 MGM. ORAL NEOSTIGMINE

In this figure the number on the tension line indicates the maximum tension developed by the muscle in grams.

and M. W. when compared to the pre-tetanus decrement. There were no consistent changes in the action potential after tetanus, *e.g.*, the action potential decrement increased after tetanus in E. R. whereas the tension decrement did not and the action potentials did not decline in M. W. but the tension did. In L. P. and M. S. W. (Figure 3) there was an increase in decrement of action potentials as well as in decrement of tension after tetanus. *After neostigmine*, there was 1) an increase in the potentiation of the initial twitch tension in three patients without an alteration in the action potential and 2) either a decrease or increase in decrement of successive twitch tensions with 3/sec. stimulation with no consistent change in the action potentials.

DISCUSSION

We interpret the results of the present study of the simultaneous electrical and mechanical activity of the adductor pollicis muscle during activity induced by supramaximal stimulation of the ulnar nerve in patients with myasthenia gravis as follows: Tension changes, especially at slow rates (3/sec.) which are much more comfortable than tetanus rates (faster than 25/sec.) are more accurate guides to the degree of muscle weakness than the electrical changes. The data lend further support to previous observations of the difficulties encountered when using the action potential pattern alone to diagnose myasthenia gravis (5).

Studies of either mechanical or electrical activity but not of both simultaneously have led to apparently conflicting conclusions. Thus Lindsley (6) reported a decrease in single twitch tension which increased with neostigmine but Pritchard (7) did not find that the single twitch tension of one myasthenia gravis patient was less than normal and Harvey and Masland (1) were unable to detect any decrease in the action potential of single induced twitches in patients with myasthenia gravis. All authors (7–9) agree that slow (about 3/sec.) repetitive contraction produces a successive decrease in tension in patients with myasthenia gravis. However, it has been demonstrated that some patients with this disorder do not have a decrement in amplitude of successive twitch potentials with induced 3/sec. stimulation (5). Furthermore, various workers (6, 7, 9) have been unable to demonstrate any change in tetanus tension until stimulation rates exceeding 80/sec. were used. But some workers have found that patients with myasthenia gravis show a progressive decrease in amplitude of successive potentials during tetanus with stimulation rates less than 40/sec. (1) and other workers have shown that although some patients with myasthenia gravis do show this electromyographic picture, others do not (5). These discrepancies cannot be explained entirely by the suggestion that various technical differences were present such as, 1) submaximal stimuli were used in some studies, 2) the interval between single stimuli and duration of tetanus varied from study to study, and 3) the severity of the disease varied from study to study. We believe that other explanations must be sought and suggest the following: 1) Myasthenia gravis, at least in some muscles of some patients, is associated with notable muscle weakness which is not accompanied by any discernible degree of neuromuscular transmission block. The findings of the present study which support this are: (a) successive twitch tensions at 3/sec. stimulation decreased significantly without any significant change in the action potentials (E. R., M. W., and M. S. W.), and (b) after an induced tetanus, successive twitch tensions at 3/sec. are initially potentiated but not sustained at the potentiated level; this abnormal finding is not accompanied by a consistent alteration in the action potential (E. R. and M. W.); 2) It is possible that post-tetanic potentiation of twitch tension, which we have shown to occur in the present study, sustains the tension during the tetanus despite a decrease in the action potentials. Brown and Burns (10) have suggested that when mammalian muscle is fatigued, there is alternation of motor unit activity during the tetanus; this explains the successive decline of the action potentials. The inactive fibers are now in the post-tetanus state even though the tetanus is continuing so that when they again contract they can contribute a greater increment of tension than they could have, had they been active throughout the tetanus. Since there is alternation of activity and inactivity of the various fibers, the tension during tetanus is sustained at the initial level because of this post-tetanic potentiation. Post-tetanic potentiation of twitch tension unassociated with facilitation of the action

potential has been shown to occur in normal human muscle and the factors which may be responsible for this phenomenon are discussed elsewhere (3).

Since the changes in muscle tension which occurred after neostigmine were not necessarily the result of changes in neuromuscular block as judged by the associated action potentials, we believe that neostigmine can have a direct effect upon the contractile ability of muscle apart from or in addition to its effect upon neuromuscular transmission. Although their data do not necessarily indicate that neostigmine has an effect on the contractile mechanism, Riker and Wescoe (11) have at least shown that neostigmine can stimulate denervated mammalian skeletal muscle. In the present study we have not excluded the possibility that the contraction was altered after neostigmine as a result of changes in the circulation to the muscle.

Although it has been shown that neostigmine administered intraarterially produces repetitive firing of normal human muscle in response to a single supramaximal stimulus (12), we were unable to demonstrate this in the present study, probably because we were using surface electrodes. It is therefore possible that repetitive firing of individual muscle fibers was present and could account for an increase in tension without altering the summed potential. If this were so, the decrease in successive twitch tensions without a decrease in action potential with 3/sec. stimulation might result from the fact that the small amounts of circulating neostigmine present from 3 to 9 hours after oral neostigmine caused repetitive firing when the muscle was stimulated through its nerve; this repetitive response would decrease with each such successive stimulus and therefore the tension of each successive twitch would decrease. If this were so, we would expect that increasing the amount of neostigmine by intramuscular injection would cause an increase in repetitive discharge of the muscle. We would then expect an increase in initial twitch tension as well as an increase in the tension of successive twitches but this occurred in only one of the four patients.

It has been shown that evoked action potentials recorded from various parts of a muscle have different forms and this might account for the differences in electrical and mechanical activity which were found in this study. It is quite true that different shaped action potentials are recorded from different parts of large muscles when surface electrodes are used (13) and from different parts of small muscles when needle electrodes are used (14). However, we do not believe this to be an important factor in the present study since the surface electrodes we used practically covered the entire belly of the adductor pollicis muscle.

It is possible that the amplitude of the action potentials may not truly reflect changes in the number of active fibers since more synchronous firing of fewer fibers could produce a potential of greater amplitude; however, in this case there should then be an alteration in the area or duration of the potential (15). Since there were no changes in potential duration or area, we believe that variations in potential amplitude did reflect variations in the number of active fibers and thus alterations in the degree of neuromuscular block. The findings of increased, decreased, or unaltered muscle tension in the absence of similar changes in neuromuscular block indicated that neostigmine can have a direct effect upon skeletal muscle of patients with myasthenia gravis. We believe that the efficacy of neostigmine must, at least in part, be the result of such a direct action.

We cannot overlook the possibility that myasthenia gravis as diagnosed by clinical signs and symptoms and frequently confirmed by the administration of neostigmine or curare (which has also been shown to have a direct effect both on mammalian [16] and human [4] skeletal muscle) may include not only patients who are weak because of a neuromuscular transmission defect but also patients who are weak because of the inability of the muscle to contract. It is apparent that the muscle as well as neuromuscular junction must be studied in order to elucidate the pathophysiology of myasthenia gravis and to obtain more effective therapeutic agents.

SUMMARY

The results of the present study show that changes in the degree of mechanical activity can occur without similar electrical changes in the adductor pollicis muscle of patients with myasthenia gravis upon supra-maximal stimulation of the ulnar nerve with single twitches, 3/sec. stimulation

and 30/sec. stimulation both before and after intramuscular neostigmine. The findings suggest 1) that changes in tension reflect more accurately the degree of muscle weakness than changes in electrical activity, 2) in some muscles of some myasthenia gravis patients the muscle weakness results from a defect in contractile ability apart from or in addition to a defect in neuromuscular transmission, 3) neostigmine is useful in some patients with myasthenia gravis because of its ability to alter the contractile processes of the muscle apart from or in addition to decreasing neuromuscular block and 4) the muscle must not be neglected in studies designed (a) to elucidate the pathophysiology of myasthenia gravis and (b) to obtain better therapeutic agents in this disorder.

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THE PHYSIOLOGICAL BASIS FOR A METHOD OF ASSAYING ALDOSTERONE IN EXTRACTS OF HUMAN URINE

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Within the past two years aldosterone, an extremely potent sodium-retaining steroid, has been isolated, structurally identified (1, 2) and subjected to numerous physiologic studies. This steroid has been isolated from adrenal tissue (3–5), from adrenal venous blood of animals (6, 7) and from human urine (8, 9).

Over the past five years there has accumulated convincing evidence that the clinical conditions which are characterized by pathological accumulation of sodium (*e.g.,* congestive heart failure, cirrhosis with ascites, and nephrosis) are also characterized by high urinary levels of sodium-retaining steroids (9–12). High levels of these steroids have also been found in the urine of healthy subjects in whom the stimulus to sodium conservation has been provided by a low-sodium diet (10). Evidence has been presented indicating that aldosterone is the principal steroid responsible for the sodium-retaining activity of such urine (8, 9).

In attempting to understand the pathogenesis of the edematous states, it would be of interest to know not only the actions of aldosterone itself but also the mechanisms governing its production. For this purpose a method of determining the quantities of aldosterone in biological fluids is required. The amounts of aldosterone appearing in human urine are so small that the measurement of this steroid by chemical methods alone is extremely difficult. For this reason it continues to be practical in carrying out clinical studies to rely on biological methods of assay.

The present studies were carried out in an attempt to characterize in detail the acute biological response to varying doses of crystalline aldosterone in the dog, and to determine whether such a response could be made the basis of a convenient assay procedure. Simultaneously, an effort was made to test the validity of the assumption that the sodium-retaining material found in urine is aldosterone by determining whether its biological actions are identical with those of aldosterone. Finally, since crystalline aldosterone is not at the present time available in sufficient quantity for general use, comparable studies were performed to determine whether desoxycorticosterone is suitable as a biological standard of reference.

We have selected the dog as an assay animal because of the ease with which detailed studies of renal function can be performed in this species. In the dog it is also easy to administer test-substances intravenously, thereby avoiding confusion arising from the fact that different steroids have different rates of absorption from extravascular tissues and that this might affect their apparent relative potencies under the conditions of a given assay, a possibility recently re-emphasized by Swingle and his co-workers (13).

METHODS

Materials

1. Crystalline aldosterone, prepared from adrenal extract by Dr. H. L. Mason of Mayo Foundation.

2. Crystalline desoxycorticosterone (free alcohol), synthesized by Ciba Pharmaceutical Products, Inc.

3. Extract of human urine. Urine was collected by three subjects receiving low sodium diets: a normal man, a patient with congestive heart failure, and a patient with ascites due to cirrhosis. All specimens were refrigerated until extracted. A total of sixty 24-hour collections were combined to provide an homogeneous pool large enough to permit repeated biological assays. The urine was acidified with HCl to pH 1 and extracted continuously for 24 hours with methylene chloride. The extract was washed with aqueous sodium carbonate, dehydrated with sodium sulfate, and evaporated to dryness under a stream of nitrogen. Each of the three steroid preparations was dissolved in absolute ethanol and stored at −7° C. throughout the study.

Assay animals

Mongrel female dogs, weighing between 15 and 20 Kg. were adrenalectomized through a single anterior ab-

dominal incision. In some, an episiotomy was performed to facilitate subsequent catheterization. Cortisone acetate was given at the time of operation, 100 mg. intragastrically and 200 mg. intramuscularly. Thereafter, 5 mg. of cortisone acetate was given intramuscularly each day. The dogs were maintained on a constant synthetic diet containing 200 mEq. of sodium and 20 mEq. of potassium daily, given as one feeding approximately 18 hours before the study was to be performed on the following day. No animals were employed until at least one week after adrenalectomy. Some had been maintained on the above regimen for more than a year.

Assay procedure

During the assay period each dog was permitted to lie or stand on a flat table. A retention catheter was inserted; the bladder was washed with distilled water (20 cc. × 4), and the washings were discarded. Thereafter, the urine was collected and the bladder washed at hourly intervals for five hours. Specimens were analyzed for sodium and potassium, using a flame photometer. At the end of the first hour, which served as a control period, the steroid or extract to be assayed was diluted with 3 volumes of water and injected intravenously.

Design of study

A crossover design was employed so that each of nine animals received, on a randomized schedule, each dose of each preparation. Six dose levels, spaced at twofold intervals, were employed for each of the three preparations (aldosterone, desoxycorticosterone, and urine extract), so that each dog received 18 treatments.

Three subsidiary studies, similarly designed, were also carried out as an empirical check on the reliability of the assay procedure.

RESULTS AND COMMENTS

Characteristics of the dose-response

In response to each of the three steroid preparations, there occurred both a decrease in excretion of sodium and an increase in excretion of potassium (Figure 1). Consistent changes were not noted during the first hour following treatment. At all dosages and for all three preparations, sodium excretion reached a minimum and potassium excretion a maximum during the second and third hours.

Of the five sodium and five potassium values so obtained for each treatment, some combination was sought which would best characterize the "sodium response" and the "potassium response" to treatment. The criterion used in selecting such a combination of data was that it serve to differentiate steroid-dosage levels with maximum precision. The second and third post-treatment hours were found to be of greatest value in this regard. The first and fourth post-treatment hours provided no additional information. Therefore, in quantitating responses we have pooled post-treatment hours two and three and discarded post-treatment hours one and four. It was found to be of further value to relate the post-treatment values to the preinjection control values. Thus *"potassium response"* equals *potassium for hours two and three* minus *potassium during control*

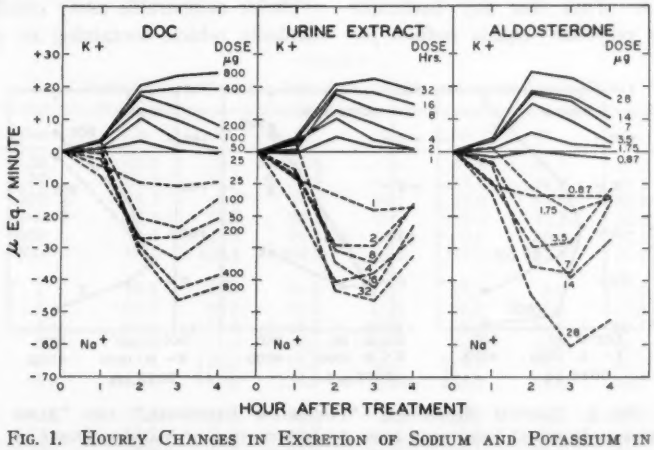

FIG. 1. HOURLY CHANGES IN EXCRETION OF SODIUM AND POTASSIUM IN RESPONSE TO DESOXYCORTICOSTERONE, URINE EXTRACT, AND ALDOSTERONE

Each line represents the average response of nine dogs. Sodium values are unweighted.

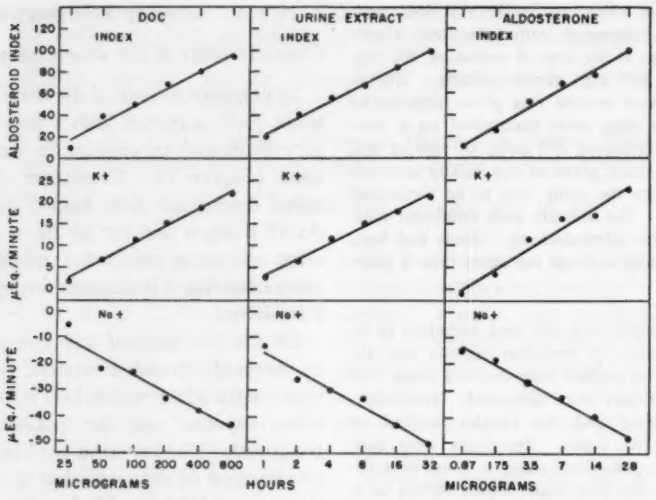

FIG. 2. "SODIUM RESPONSES," "POTASSIUM RESPONSES," AND "ALDOSTEROID INDEXES" RESULTING FROM ADMINISTRATION OF VARYING DOSES OF DESOXYCORTICOSTERONE, URINE EXTRACT, AND ALDOSTERONE

Each dot represents the average response of nine dogs. Logarithmic dosage scale.

hour, all values expressed as μEq. per minute. For the sodium response a further significant increase in precision was obtained (as shown by the analysis of covariance) by using, not the observed control, but rather what we have termed a weighted control value. The weighted control value derived for this set of data was 54 + 0.4 (observed control). Thus, for any particular treatment, *"sodium response"* equals *sodium for hours two and three* minus [*54 + 0.4 (control sodium)*], expressed as μEq. per minute.

The average "sodium responses" and "potassium responses" are plotted against doses of all three preparations in Figure 2. It is apparent that each response is a linear function of the logarithm of dose over the entire 32-fold range.

Under comparable assay conditions, when the alcoholic vehicle containing no steroids was ad-

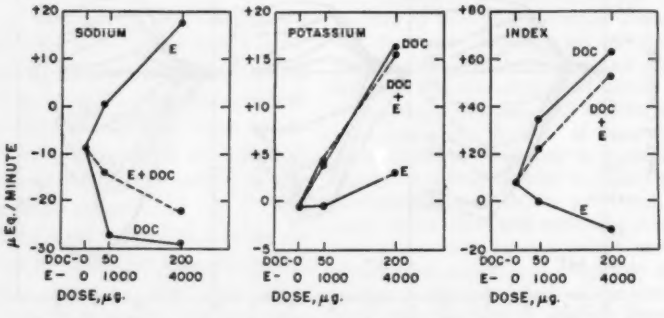

FIG. 3. "SODIUM RESPONSES," "POTASSIUM RESPONSES," AND "ALDOSTEROID INDEXES" RESULTING FROM ADMINISTRATION OF VARYING DOSES OF DESOXYCORTICOSTERONE ALONE, CORTISONE (E) ALONE AND OF THE SAME DOSES OF DESOXYCORTICOSTERONE PLUS CORTISONE

Each dot represents the average response of six dogs.

ministered (Figure 3), there was a slight tendency for the excretion of both sodium and potassium to decrease during successive hours.

For aldosterone, the slope of the line relating sodium response to dose was -22.5 μEq. per minute per tenfold dilution of aldosterone. For desoxycorticosterone (DOC) it was -23.2, and for urine extract it was -22.8. The standard error of each slope was 2.9. These slopes were not significantly different from one another. Relating potassium responses to dose of each preparation, the average slope for aldosterone was 15.7, for DOC 13.5, and for the urine extract 12.3. The standard error of each slope was 1.5. Once again, these slopes did not differ significantly.

Having established the parallelism of the dose-response curves for the three steroid preparations, we may proceed to determine their ratios of potency. The ratio of potency $\frac{\text{aldosterone}}{\text{DOC}}$ when measured in terms of sodium-retaining activity was 36-fold and when measured in terms of potassium-losing activity 26-fold. Ninety-five per cent confidence limits on these estimates were 24 to 56 and 18 to 37, respectively. The ratio of potency of $\frac{\text{aldosterone, } \mu\text{g.}}{\text{urine extract, hr.}}$ when measured in terms of sodium-retaining activity was 0.87 and when measured in terms of potassium-losing activity 1.03. Ninety-five per cent confidence limits were 0.56 to 1.34 and 0.72 to 1.47, respectively. Clearly, the relative potencies of these steroid preparations were essentially the same whether they were compared on the basis of sodium retention or potassium loss.

The experience of Desaulles, Tripod, and Schuler (14) suggesting that aldosterone is only five times as potent as DOC in its influence on potassium excretion, whereas it is thirty times as potent as DOC in its effect on sodium excretion, has no parallel in the present study.

Since the potassium and sodium responses give essentially the same estimates of relative potency, it is permissible to combine these responses into a single index, the criterion of combination being solely that it yield the maximum information about steroid dosage which can be derived from a given set of responses. Such a combination, which we have termed the aldosteroid index, is given by the method of discriminant functions (15).

As estimated from the experiment summarized in Figures 1 and 2,

Aldosteroid index $= 2.3$ (*"K response"*) $-$ *"Na response."*

We show in Table I the index values for the nine dogs, six dose levels and three materials included in the present study. The average index for nine dogs is represented for each treatment in Figure 2.

Recalculation of the ratios of potency of the three steroid preparations indicates that by the present method 1 μg. of aldosterone exhibited ac-

TABLE I

Aldosteroid index for nine dogs, three steroids each at six dose levels

	Dog	A	B	D	E	G	J	K	N	R
DOC (μg.)	25	52.4	29.2	15.8	−5.6	19.8	14.2	12.2	−14.9	−33.4
	50	60.2	41.9	30.2	10.6	83.9	50.8	28.2	14.2	23.6
	100	64.2	44.6	54.7	38.2	83.0	63.6	37.9	23.3	34.9
	200	85.6	78.5	80.2	48.9	115.0	71.7	50.0	48.2	37.2
	400	91.7	124.6	94.0	49.4	97.0	80.8	71.4	73.8	47.6
	800	90.0	136.4	104.0	63.3	123.0	108.4	81.0	63.7	81.3
Urine extract (*hr.*)	1	49.1	25.2	41.2	13.7	25.3	8.0	16.8	20.2	−13.8
	2	64.0	46.2	49.2	12.6	58.3	66.9	42.0	32.4	2.1
	4	83.0	75.8	65.2	25.0	59.8	86.0	62.8	29.6	24.1
	8	98.0	113.6	76.0	15.8	73.3	92.0	66.3	45.7	29.7
	16	117.6	116.6	97.0	38.2	119.2	93.2	82.1	55.8	49.8
	32	121.8	147.4	134.9	53.6	133.2	88.8	76.2	102.0	41.3
Aldosterone (μg.)	0.875	28.0	25.4	34.3	2.2	25.2	6.5	−5.2	9.6	−8.3
	1.75	53.8	41.0	12.5	14.2	40.8	27.6	25.1	13.0	21.6
	3.50	68.6	88.4	67.8	21.6	89.1	53.8	33.4	33.8	32.4
	7.00	97.4	130.0	66.1	34.6	83.6	84.5	64.7	50.0	27.8
	14.	86.6	115.8	87.1	44.0	109.3	98.0	82.5	43.7	51.8
	28.	107.4	121.8	125.4	41.0	167.8	103.4	114.0	88.0	50.2

TABLE II

Analysis of variance of aldosteroid index

	Source of variation	Degrees of freedom		Mean square
A	Dogs	8		9,030
	Dose levels:	5		
B	Regression		1	127,859
C	Deviation from regression		4	238
D	Steroids	2		391
	Interactions			
	Dog × dose level:	40		
E	Difference in regressions		8	900
F	Variation about individual regressions		32	147
G	Dog × steroid	16		209
	Dose level × steroid:	10		
H	Difference in regressions		2	203
I	Variation about individual regressions		8	129
J	Dog × dose level × steroid	80		168
		161		

tivity comparable to that of 29.9 μg. (24.2 to 36.9) (95 per cent confidence limits) of DOC and to that of 0.96 hour (0.78 to 1.19) of urine extract. The ratio of potency of aldosterone to DOC is of the same order as that reported previously by Gross and Gysel (16), by Mach and associates (17), and by Kekwick and Pawan (18), all using different methods.

Some idea of the relative utility of alternative methods of analyzing assay data can be gained by comparing their respective "lambda" values.[1] The present method of assay and analysis gives values of λ in the neighborhood of 0.24. This implies that in estimating ratios of potency for two preparations, six observations on each preparation would be sufficient to establish with 95 per cent confidence that a given estimate is not less than one-half nor more than twice the true value.

The gain in precision which is achieved by using the "aldosteroid index" rather than either of the individual responses is shown by comparison of the corresponding values of λ: 0.24 for the index, 0.37 for the potassium response, and 0.48 for the sodium response. This implies that if one were to use the latter metameters instead of the "index" one would require from two to four times as many observations to derive the same amount of information.

Table II presents an analysis of variance of the aldosteroid indexes. The square root of the mean square for the highest order interaction (dog by dose by steroid) supplies an estimate of the standard deviation of the indexes that would be obtained from repeated measurements on the same dog at a single dose level for a single steroid; *i.e.*, it provides the estimate of random error. Three of the mean squares (lines A, B, and E) significantly exceed this estimate of error at the .001 level; the remainder do not differ significantly from random error at the .05 level. We conclude from this analysis, first, that dose-response curves for individual dogs differ in both level and slope (lines A and E). Use of the crossover design eliminates this source of variability and, since we are interested in the relative potencies of the materials tested rather than the characteristics of the population of assay subjects, the use of a crossover design increases the precision of the assay. Second, we conclude that the differential response to different materials is the same for all dogs (line G), so that as far as the evidence of this experiment goes, each dog supplies essentially the same estimates of potency. Third, the dose-response lines for the three different steroids are parallel (line H). Fourth, there are no significant departures from linearity (lines C, F and I). Fifth, the experiment was designed so that if there were significant day effects, their main effects, would be balanced out. The mean square for days, 203, (not shown in Table II), is not significantly above random error, thus indicating that dogs make a satisfactory return to control status within 24 hours after a test.

[1] "Lambda" = standard deviation ÷ slope.

Reproducibility of assay results

To what extent will the aldosteroid index provide useful results when used in an experiment other than the one it was derived from? We summarize in Table III the average values of the aldosteroid indexes provided by each of four independent experiments. At the foot of the table we show the standard deviations, slopes and values of λ derived from each experiment. The values of λ vary from 0.19 to 0.26 and indicate that the utility of the aldosteroid index is not confined to the experiment from which it was derived.[2] The variation from experiment to experiment in the absolute levels of the indexes reflects differences in responsiveness of different dogs. The five dogs used in experiment IV were also used in experiment III. Three of these five dogs showed significant declines in responsiveness over the period from October to January. The biological significance of these declines is not at present known, but in view of the unchanged magnitude of the standard deviation and slope, it would appear to have no effect upon the precision of the present assay.

[2] For experiments 1 and 2 the standard deviation was estimated from the dog-dose interaction, which may, in view of the results of Table II, provide an overestimate.

Interference by contaminating steroids

Earlier experience indicated that under the conditions of this assay procedure hydrocortisone and cortisone in doses of more than 1 mg. caused increases in excretion of sodium (19). Data presented in Figure 3 illustrate the manner in which this acute sodium-losing action of cortisone can modify results obtained by the present method. Fortunately the amounts of cortisone or hydrocortisone required to produce this interference are so large that they would probably never be encountered in extracts of human urine except during treatment with large doses of 17-hydroxycorticosteroids or ACTH or in patients with Cushing's syndrome. The extract of urine employed in the present study contained no more than 3 μg. of 17-hydroxycorticosteroids per "hour" of extract, as estimated by the Porter-Silber reaction. Contamination of this degree is far below that necessary to modify assay results. The close similarity between the responses to this urine extract and responses to crystalline aldosterone has been interpreted as indicating that biologically detectable contamination did not occur. Whenever heavy contamination of an extract with 17-hydroxycorticosteroids is a possibility, however, it would appear advisable to remove them before proceeding with the bioassay.

TABLE III

Aldosteroid indexes at different dose levels and for different steroids in four independent experiments

	Exp. I	Exp. II	Exp. III			Exp. IV		
	April	July	October			January		
Dose level μg. DOC*	DOC	DOC	DOC	Aldosterone*	Urine extract*	DOC	Aldosterone*	Urine extract*
0	14.9	22.1	—	—	—	—	—	—
25	—	—	10.0	13.1	20.6	—	—	—
50	39.6	59.5	38.2	27.7	41.5	28.4	20.3	34.1
100	66.3	80.5	49.7	54.2	56.8	—	—	—
200	88.1	105.4	68.4	71.0	67.3	63.8	63.2	71.9
400	99.2	124.3	81.2	79.9	85.5	—	—	—
800	114.2	131.6	94.7	102.1	99.9	—	—	—
1,600	112.5	—	—	—	—	—	—	—
Standard deviation (s)	14.1	16.0		13.0			11.9	
Slope (b)	60.5	62.5		54.7			63.0	
$\lambda = s/b$	0.23	0.26		0.24			0.19	

Exp. I—Cross-over on 7 dogs, each tested for 7 days.
Exp. II—Cross-over on 6 dogs, each tested for 6 days.
Exp. III—Cross-over on 9 dogs, each tested for 18 days (individual results in Table I).
Exp. IV—Cross-over on 5 dogs, each tested for 8 days.
* Responses to 0.875 μg. aldosterone and 1 hr. of urine extract are on line 25 μg. DOC.

SUMMARY

A method for assaying aldosterone and like steroids has been described. This method entails the use of adrenalectomized female dogs, maintained on a high sodium diet. The effects of varying doses of aldosterone on sodium and potassium excretion have been presented. Administration of crystalline aldosterone results in a rise in potassium excretion and a fall in sodium excretion, both of which are rectilinear functions of the log-dose of aldosterone over the range from 1 to 30 micrograms. An "index" has been derived for the purpose of reducing the complex response to a single dimension, while achieving maximum precision ($\lambda = 0.24$) in differentiating dosage levels. With this method the response to aldosterone closely resembles the response to crystalline desoxycorticosterone which is approximately 1/30 as potent as aldosterone. Extracts of urine from patients with congestive heart failure, cirrhosis with ascites, and from healthy subjects receiving a low sodium diet, have been found to possess activity indistinguishable from that of aldosterone. The responses to aldosterone are easily distinguishable from the responses to hydrocortisone-like steroids by the present method.

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A MODIFICATION OF THE KROGH CARBON MONOXIDE BREATH HOLDING TECHNIQUE FOR ESTIMATING THE DIFFUSING CAPACITY OF THE LUNG; A COMPARISON WITH THREE OTHER METHODS[1]

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There are at present four methods for assessing the pulmonary diffusing capacity (D), one using O_2 (D_{O_2}) and three using CO (D_{CO}) namely, the O_2 method of Lilienthal, Riley, Proemmel, and Franke (1), the steady state CO method of Filley, MacIntosh, and Wright (2) and the CO breath holding technique of Krogh and Krogh (3, 4) as modified by some of the authors (5), all of which provide estimates of the pulmonary diffusing capacity, and finally the fractional CO uptake of Bates (6) which gives an index of pulmonary diffusing capacity. This is a report of a cooperative research effort to compare these methods as nearly simultaneously as possible on six patients and one normal subject. Measurements were made over the extremes of pulmonary diffusing capacity, from a patient with a severely decreased pulmonary diffusing capacity at rest (Subject 5) to a normal subject during exercise (Subject 1).

METHODS

A. *Steady state D_{O_2} method*

This method requires the simultaneous sampling of expired gas and arterial blood with the subject breathing first air and then a low concentration of O_2. Analysis of the alveolar-arterial P_{O_2} gradient under these two conditions makes possible the estimation of the mean alveolar-capillary P_{O_2} difference, which is divided into the O_2 uptake to give D_{O_2}. This technique has been adequately described in detail before (1, 7, 8), the only significant alteration in previous procedure was in the gas mixtures breathed. For an initial period of equilibration of 5 to 12 minutes the subject inspired a predetermined O_2 and N_2 mixture from a compressed gas tank. At the end of this period, the patient was suddenly switched to inspire a gas mixture containing the same percentages of O_2 and N_2 and in addition approximately 0.1 per cent CO. After breathing this mixture for a further 1 to 2 minutes, the collection period was started as usual, (1). This procedure permitted simultaneous steady state estimates of D_{O_2} and D_{CO}, with a minimal total CO uptake. Although all but two of the seven individuals were studied during exercise, we do not know whether the values of D_{O_2} and steady state D_{CO} are *maximal diffusing capacities* (7) or not.

B. *Steady state D_{CO} method*

This procedure has been described in detail by Filley, MacIntosh, and Wright (2) and will not be fully discussed here. In general it consists of establishing steady state conditions while breathing a mixture containing 0.1 to 0.15 per cent CO, and measuring the inspired and expired CO and CO_2 concentrations, plus the arterial P_{CO_2}. From the ventilation and the CO concentrations, the CO uptake can be calculated. As in the method of Lilienthal, Riley, Proemmel, and Franke (1), arterial P_{CO_2} is considered equal to mean alveolar P_{CO_2} and, along with the values above, is used to calculate alveolar P_{CO}.[5] This value for effective alveolar P_{CO} is divided into the CO uptake to give D_{CO}. Although Filley, MacIntosh, and Wright used a 6-minute preliminary period breathing the CO mixture (2), Bates (6) and ourselves (9) found that 1 to 2 minutes sufficed to reach a sufficiently steady state under these circumstances. Analyses of CO concentration in the various gases were made with an infra red gas analyzer,[6] instead of by the chemical method used by Filley, MacIntosh, and Wright (2).

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[5] A simple version of the equation used is:

$$\text{alveolar } P_{CO} = \text{inspired } P_{CO} - \frac{\text{arterial } P_{CO_2}}{\text{expired } P_{CO_2}} (\text{inspired } P_{CO} - \text{expired } P_{CO}).$$

[6] Liston-Becker Co., Stamford, Conn.

C. *Fractional CO uptake*

This is

$$\frac{\text{CO absorption in ml./min.}}{\text{CO inspired in ml./minute (corrected for instrumental dead space)}}.$$

The data were available from the determinations of D_{CO} by the steady state technique, and were not collected in a separate procedure as described by Bates (6).

D. *Breath holding technique for D_{CO}*

We regarded this study as an opportunity to compare the modified Krogh breath holding technique for measuring pulmonary diffusing capacity with the other methods and to obtain simultaneous rather than consecutive comparisons of D_{CO} steady state with D_{O_2}. We have previously published a detailed analysis of a modification of the Krogh breath holding technique, which utilizes 10 per cent He along with a small concentration of CO in the inspired mixture to permit correction for the effects of uneven distribution of inspired gas throughout the lung upon the calculation of D_{CO} (5). In addition to the effects of uneven inspired gas distribution upon the calculated D_{CO}, there is the effect of duration of breath holding: calculated D_{CO} decreases with the duration of breath holding (5). Nevertheless, it seemed worthwhile to determine whether careful standardization of the test might provide clinically useful values for D_{CO}. The desirability of developing a quick and repeatable clinical test with minimal discomfort to the patient justified the inclusion of the single breath procedure. We have arbitrarily chosen a 10-second period for breath holding because all the patients studied were able to hold their breath this long, because the resulting change in alveolar CO concentration was large enough to measure accurately, and because the values for D_{CO} at 10 seconds are reasonably comparable to those in the literature. Although a series of 5 to 9 estimations of D_{CO} was made on each individual at different periods of breath holding up to his limit, or to 60 seconds, only those values obtained with breath holding periods from 9 to 15 seconds

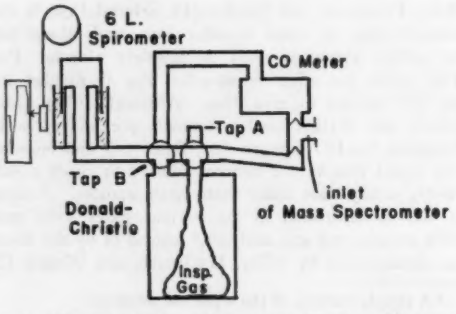

FIG. 1. DIAGRAM OF THE APPARATUS USED TO MEASURE D_{CO} BY THE BREATH HOLDING TECHNIQUE

Tap A and Tap B were used in making up gas mixtures.

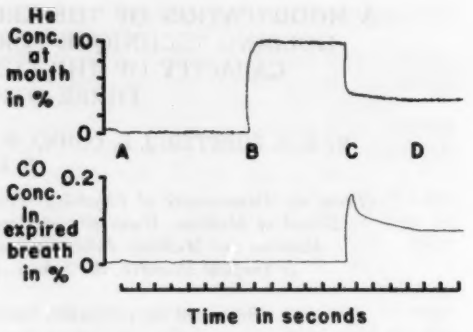

FIG. 2. RECORD FROM PATIENT 2 SHOWING THE He CONCENTRATION IN THE GASES AT THE MOUTH AND THE CO CONCENTRATION IN EXPIRED GAS AS USED IN THE DETERMINATION OF D_{CO} BY THE BREATH HOLDING TECHNIQUE

are included. A variation of the duration of breath holding between these particular limits caused changes in the calculated D_{CO} of less than 10 per cent judging by the individual graph of expired alveolar CO concentration versus time of breath holding. The range of 9 to 15 seconds represents extremes of actual breath holding times in inexperienced patients attempting to hold their breaths for the standard 10 seconds. The slow rate of expiration in some patients made the actual breath holding time greater than 10 seconds more often than it was less than 10 seconds.

The apparatus (Figure 1) and method of measuring the breath holding D_{CO} differed from that described previously (5) mainly in that the expired breath went directly through a low flow resistance sample chamber in the infra red CO analyzer and was not collected in a bag for later analysis. Concentration of He at the mouth was analyzed with a continuously sampling mass spectrometer.[7] Both the expired CO and He concentrations were recorded through DC amplifiers on a Brush magnetic pen ink writer system. Although the He concentration was recorded with a 90 per cent response time of 0.06 second, the slower response time of the infra red analyzer (90 per cent in 0.4 second) combined with the lag in time for the washout of the actual analysis chamber (25 ml.) and associated tubing (100 ml.) made it difficult to compare the two records while expiration was in progress. Therefore, the He and CO concentrations were compared at the end of expiration, after the infra red CO analyzer had had time to respond completely to the expired gas that remained in the chamber.

In performing the breath holding D_{CO} determination the patient sat quietly wearing a nose clip. At the end of a normal expiration, he took the mouthpiece in his mouth, expired to his residual volume, and then inspired, as rapidly as possible, a maximal volume from the bag of the Donald-Christie apparatus which contained a

[7] Consolidated Engineering Corp., Pasadena, California.

mixture of 0.3 per cent CO, 10 per cent He, 20 per cent O_2 and 70 per cent N_2. He held his breath for approximately 10 seconds and then expired, again as rapidly as possible, a volume of 1 liter or more.

A record of He concentration at the mouth and expired CO concentration is shown in Figure 2. At the beginning of the record there is a slight contamination of He in the mouth piece which decreases to zero with the start of patient's expiration (A). At B, the patient started to inspire and the He concentration at the mouth rose rapidly to 10 per cent. Six seconds after the start of inspiration the patient started to expire (C) and the He concentration at first fell abruptly to 4.4 per cent and then continued to fall throughout the rest of the expiration in a downward sloping "alveolar plateau." At D, 9.8 seconds after the start of inspiration, the patient completed his expiration; the He concentration remained constant thereafter, and the CO record leveled off less than one second later. The output of the CO meter between A and B, when normal expired gas is in the analysis chamber, is considered zero CO concentration as the patient had previously inspired no CO. The water vapor and CO_2 in the expired gas at this point provide an automatic correction for the interference of these gases with the CO analysis of the expired alveolar sample at D. During inspiration (B) no CO appears in the analyzer because of the valves in the mouthpiece. At C, expired gas containing CO is blown through the infra red analyzer, the initial peak being inspired gas from the respiratory and instrumental dead spaces. At D, the final expired gas is trapped in the analyzer. This CO concentration and the corresponding final expired alveolar He concentration are used to calculate the pulmonary diffusing capacity.

In the example illustrated in Figure 2, the concentration of inspired CO was 0.302 per cent (dry) and of inspired He 10 per cent (dry). At point D, the final CO concentration was 0.069 per cent (BTS) and that of He was 3.52 per cent (BTS). Since He is practically insoluble in the blood relative to CO, the decrease in the He concentration from 10 per cent in the inspired gas to 3.52 per cent in the expired alveolar gas is entirely a result of its dilution by the residual gas in the lung at the start of inspiration. The inspired CO concentration must have been decreased by dilution alone to precisely the same degree as the inspired He. Furthermore, the gas within those regions of the lung where significant CO absorption takes place, must be mixed within at least a tenth of a second after inspiration (10). Therefore, the initial CO concentration, before any absorption into the blood had taken place, of that particular aliquot of the expired gas which was in the infra red analyzer chamber at the end of expiration, must have been (3.52/10.0) × 0.302 per cent CO, or 0.106 per cent BTS. This calculation of the initial CO concentration in a particular expired gas sample will be true whether the inspired gas was evenly or unevenly distributed and whether the expired sample came from one or a great many different alveoli. According to the equation of Krogh and Krogh (3),

$$D_{CO} = \frac{\text{alveolar volume (STPD)} \times 60}{\text{time in seconds} \times (B\text{-}47)} \times \text{Ln}\left[\frac{\text{initial expired alveolar CO concentration}}{\text{final expired alveolar CO concentration}}\right]. \quad (2)$$

The alveolar volume equaled the sum of the volume inspired (1280 ml. STPD) plus residual volume (3780 ml. STPD, by the method of Darling, Cournand, and Richards [11]) or 5060 ml.: B-47 was 713 mm. Hg: the time of breath holding, actually the time from the start of inspiration until the end of the expiration, was 9.8 seconds. Thus the breath holding D_{CO} equaled

$$\frac{5{,}060 \times 60}{9.8 \times 713} \text{Ln}\,[0.106/0.069] = 18.5 \text{ ml./min./mm. Hg.}$$

These measurements of D_{CO} by the breath holding method were made the same day as the measurements of D_{O_2} and the steady state D_{CO} in the majority of patients.

It can be seen in Figure 2 that the expired alveolar He concentration decreases during the course of a single expiration, which means that the proportion of inspired gas in the expired alveolar gas decreases during the expiration. In the technique originally described by Krogh (4), two alveolar samples were collected, the initial one from the first part of an expiration, and the other from a later part of the same expiration, after an intervening breath holding period of 10 seconds' duration. Since the difference between the CO concentrations of the two samples was considered wholly a result of diffusion of CO into the blood, an incorrect value of D_{CO} was obtained.[8] We avoid this particular difficulty by using the dilution of the inspired He to calculate the initial CO concentration of the alveolar sample.

E. *Other pulmonary function studies*

Determinations of lung volumes, including residual volume by the method of Darling, Cournand, and Richards (11), maximum breathing capacity, per cent of the vital capacity expired in one second, uneven ventilation estimated by the method of Cournand, Baldwin, Darling, and Richards (12) and by the single breath test of Comroe and Fowler (13), were also carried out on each patient.

SUBJECTS

The subjects were chosen so as to give as large a range of values for diffusing capacity of the lungs as possible. Pt. 1 was a healthy young male with no evidence of pulmonary disease. Pt. 2 had a history, physical findings and X-ray examination compatible with a

[8] Since the apparent rate of disappearance of CO is greater than the actual, under these circumstances, the Krogh technique will lead to an overestimate of D_{CO}. If we had used the two-stage expiration technique in the example given above, we would have obtained a D_{CO} of approximately 24 ml. per min. per mm. Hg, instead of 18.5 ml. per min. per mm. Hg.

TABLE I

Results of pulmonary function studies *

Subject	Diagnosis	Sex	Age *yrs.*	Height *inches*	Weight *pounds*	Surface area M^2	VC *ml.*	IC *ml.*	ER *ml.*	RV *ml.*	TLC *ml.*	RV/TLC %†	MBC *L./min./M^2*	% VC in 1 sec. %‡	Alv. N_2 after 7 min. %§	N_2 single breath test %‖
1	Normal	M	27	66	160	1.81	4,260 (4,250)	2,900 (3,380)	1,380 (845)	1,592 (1,070)	5,852 (5,320)	27	107 (72)	88	0.4	0.6
2	Emphysema	M	54	64½	185	1.89	1,440 (3,940)	910 (2,995)	577 (985)	4,590 (1,380)	6,130 (5,320)	75	13 (58)	30	<2.5	10.5
3	Silicosis	M	42	74	172	2.02	4,640 (4,750)	2,980 (3,800)	1,140 (950)	5,127 (1,420)	9,767 (6,190)	52	64 (86)	73	0.9	2.6
4	Chronic bronchitis	M	63	66	107	1.52	1,735 (3,860)	1,350 (2,895)	566 (905)	4,397 (1,740)	6,132 (5,600)	72	38 (54)	47	2.7	7.4
5	Sarcoidosis	F	45	66	140	1.66	1,485 (2,925)	1,155 (1,950)	333 (975)	1,719 (895)	3,204 (3,810)	54	57 (50)	86	<2.5	3.0
6	Sarcoidosis	F	39	66	134	1.67	1,685 (3,350)	1,425 (2,230)	267 (1,120)	1,926 (1,030)	3,611 (4,380)	53	29 (53)	79	<2.5	
7	Mitral commissurotomy (postoperative)	M	41	68	196	2.00	4,270 (4,320)	3,140 (3,456)	1,112 (864)	2,042 (1,320)	6,312 (5,640)	32	78 (65)	71	1	1.3

* Figures in brackets are predicted normal values.
† Normal value <30 per cent.
‡ Normal value >83 per cent.
§ Normal value <2.5 per cent.
‖ Normal value <2.0 per cent.

Symbols for lung volumes: VC is vital capacity; IC is inspiratory capacity; ER is expiratory reserve; RV is residual volume; TLC is total lung capacity; MBC is maximum breathing capacity.

diagnosis of pulmonary emphysema, which was borne out by his pulmonary function tests. He also had a good deal of bronchospasm and, in fact, it was found necessary to use bronchodilators to make him comfortable during the diffusion capacity measurements. Pt. 3 had a history of exposure to silica, complained of dyspnea, and had physical and X-ray findings associated with silicosis. Pt. 4 had a long history of chronic bronchitis and physical and X-ray findings compatible with that diagnosis. Pts. 5 and 6 both had sarcoidosis, with the typical history and chest X-ray findings associated with that disease, and fitted the syndrome of "alveolar-capillary" block. Pt. 7 although he had had a mitral commissurotomy about 6 months prior to these studies, was essentially normal as far as his pulmonary function was concerned.

RESULTS

The vital statistics and pulmonary function studies on each subject are in Table I. D_{O_2}, steady state D_{CO}, fractional CO uptake, D_{CO} breath holding, and pertinent data for each subject are presented in Table II. The reader is referred to the published discussions of these methods (1, 2, 5–8) for a detailed explanation. There are two different values for D_{CO} steady state and fractional CO uptake to compare with each value of D_{O_2}, because it is possible to get the CO values while breathing both the air and the low O_2 mixtures which are necessary for each value of D_{O_2}. These data are also shown in Figure 3; average D_{CO} breath holding, D_{CO} steady state and fractional CO uptake measurements are plotted on the ordinates against the D_{O_2} values on the common abscissa.

DISCUSSION

A. *Comparisons of D_{O_2}, D_{CO} steady state, D_{CO} breath holding and fractional CO uptake*

The relations between the different estimates of pulmonary diffusing capacity are best seen in Figure 3. The lowest graph is a plot of D_{CO} breath holding against D_{O_2}. There is a definite increase in D_{CO} breath holding as D_{O_2} rises. The dashed lines in the lower two graphs represent the relation that would hold between D_{CO} and D_{O_2} if the only difference between the two measurements lay in the different molecular weights and solubilities of O_2 and CO and if the pulmonary membrane were chiefly saline (3). There are, however, many differences in the physiologic bases of D_{CO} breath holding and D_{O_2}. For example, if the standardized time of breath holding were taken to be 20 seconds instead of 10, D_{CO} breath holding would decrease (5), as noted earlier in this paper. In addition, recent work of Pappenheimer, Renkin, and Borrero (14) suggests that the lipid solubility of gases may be the most important fac-

TABLE II *

Simultaneous D_{O_2}, D_{CO} steady state and fractional CO uptake measurements, and resting D_{CO} breath holding measurements on seven individuals

Subject	Exercise: Rate *m.p.h.*	Exercise: Grade %	$F_{I_{O_2}}$ %	$\dot{V}_E$ *L.(STPD)/min.*	Resp. rate *per min.*	$\dot{V}_{O_2}$ *ml.(STPD)/min.*	R	$P_{a_{CO_2}}$ *mm. Hg*	$P^{\bar{a}}_{A_{O_2}}$ *mm. Hg*	$P_{a_{O_2}}$ *mm. Hg*	$P^{\bar{a}}_{A_{O_2}} - \bar{P}_{C_{O_2}}$ *mm. Hg*
1	2.5	6	20.93	32.9	44	1,469	0.81	38	102	92	17
	2.5	6	12.72	34.6	46	1,096	0.80	35	47	38	17
2	0	0	20.93	10.2	19	337	0.74	45	94	78	13
	0	0	13.97	9.8	16	282	0.90	43	51	44	13
3	1.3	6	20.93	34.5	26	1,342	0.89	39	104	92	25
	1.3	6	12.72	48.6	35	1,405	0.95	34	54	37	25
4	1.3	6	20.93	30.0	34	956	0.80	46	93	75	26
	1.3	6	13.97	25.6	30	619	0.91	36	59	42	26
5	1	0	20.93	24.2	32	604	0.80	35	105	60	43
	1	0	13.97	35.8	50	510	1.02	28	70	31	43
6	0	0	20.93	13.2	40	301	0.88	41	102	86	21
	0	0	13.97	13.3	46	230	0.89	36	59	46	21
7	1.5	0	20.93	21.0	13	961	0.85	37	108	95	15
	1.5	0	12.72	22.3	12	952	0.78	30	54	49	15

TABLE II—*Continued*

Subject	V_D/V_T %	$\dot{Q}_{VA}/\dot{Q}_T$ %	D_{O_2} *ml.* / *min.* × *mm. Hg*	F_{ICO} %	CO uptake *ml./min.*	P^eA_{CO} *mm. Hg*	Fractional CO uptake %	D_{CO} steady state *ml.* / *min.* × *mm. Hg*	D_{CO} breath holding at rest, breathing air *ml.* / *min.* × *mm. Hg*
1	24	3	65	0.092	12.6	0.24	46	52	37, 41
	43			0.089	11.2	0.17	40	66	
2	54	8.5	22	0.094	3.4	0.05	43	71	13, 13, 18
	50			0.107	3.2	0.20	36	16	
3	32	4	56	0.098	10.7	0.34	34	32	20, 31
	38			0.088	12.2	0.30	30	41	
4	50	8	24	0.148	10.0	0.46	25	22	12
	47			0.098	6.7	0.26	29	26	
5	53	20	12	0.095	2.7	0.47	13	6	7, 7, 8, 9
	60			0.088	2.0	0.50	7	4	
6	54	6.5	11	0.138	4.1	0.34	28	12	14
	53			0.083	2.9	0.13	36	22	
7	21	3.5	63	0.154	16.7	0.35	54	48	22
	17			0.067	7.3	0.17	50	42	

* F_{IO_2} is dry inspired O_2 concentration in %.
$\dot{V}_E$ is expired minute ventilation in liters STPD/min.
V_{O_2} is the O_2 consumption in ml. STPD/min.
R is the respiratory exchange ratio.
Pa_{CO_2} is the arterial CO_2 tension in mm. Hg measured directly.
$P^eA_{O_2}$ is the calculated effective alveolar O_2 tension in mm. Hg.
Pa_{O_2} is the arterial O_2 tension in mm. Hg measured directly.
Pc_{O_2} is mean capillary O_2 tension in mm. Hg.
V_D/V_T is dead space volume/tidal volume in %: normally less than 25%.
$\dot{Q}_{VA}/\dot{Q}_T$ is the "physiological" shunt of mixed venous blood across the lung over the total capillary blood flow in %. Called "percentage venous admixture." Normally less than 6%.
D_{O_2} is pulmonary diffusing capacity for O_2 in ml. STPD/min./mm. Hg.
F_{ICO} is dry inspired CO concentration in %.
P^eA_{CO} is the calculated effective alveolar CO tension in mm. Hg corrected for the increase in COHb during the experiment.
D_{CO} is pulmonary diffusing capacity for CO, either steady state or breath holding, in ml. STPD/min./mm. Hg.

tor in their diffusion through the pulmonary membrane, which would change the theoretical relation between D_{CO} and D_{O_2}. Patients 2 and 6 were studied at rest both for D_{CO} breath holding and D_{O_2}. Patients 4 and 5, whose D_{CO} breath holding was measured at rest and whose D_{O_2} during exercise, had diagnoses of long standing chronic bronchitis and sarcoidosis, respectively. It is apparent that these patients were not able to increase their pulmonary diffusing capacity on exercise (7) to the same extent as normal subjects, and for this reason there is less difference between the breath holding values determined at rest and the D_{O_2} values obtained during exercise. Patients 1, 3, and 7 on the other hand, who were considered to be in better physical condition than the others, did presumably increase their D_{O_2} on exercise which displaced their points to the right. It should be noted that the D_{O_2} measurements on relatively healthy individuals, are more successful technically when done during exercise. At the time this study was done, we were not able to make D_{CO} breath holding measurements during exercise. Presumably, D_{CO} breath holding would have increased with exercise, displacing the values for patients 1, 3, and 7 upwards, as more recent studies have shown (15). All in all the findings are not unreasonable in view of the circumstances under which they were obtained.

The middle graph in Figure 3 is a plot of simul-

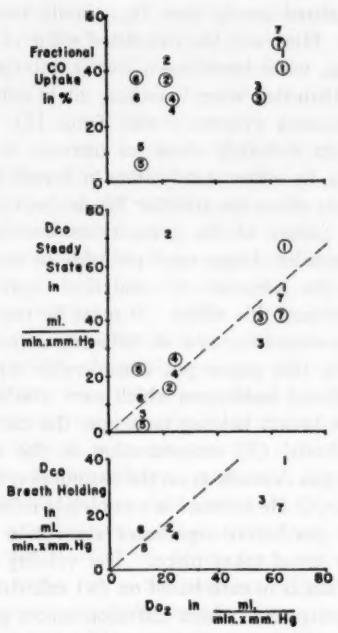

FIG. 3. FRACTIONAL CO UPTAKE, D_{CO} STEADY STATE AND D_{CO} BREATH HOLDING PLOTTED AGAINST D_{O_2}

The numbers refer to the particular subject; a circle about a number indicates that value was obtained breathing an O_2 concentration less than 20.93 per cent. The dashed lines are the theoretical relationships between the pulmonary diffusing capacity for O_2 and that for CO; $D_{O_2} = 1.23\ D_{CO}$. Individuals 1, 3, 4, 5, and 7 were studied during exercise, except for the D_{CO} breath holding which was measured at rest in all subjects. Individuals 2 and 6 were studied in rest in all tests.

taneous measurements of D_{CO} steady state against D_{O_2}. In this comparison, the level of exercise was obviously the same during both tests. These two methods are analogous in that both are performed during a steady state and, in both, alveolar tensions (P_{O_2} or P_{CO}) are calculated from the Bohr equation, assuming that arterial P_{CO_2} is a good estimate of mean alveolar P_{CO_2}. The value of D_{CO} steady state is sensitive to errors in arterial P_{CO_2} particularly when the fractional uptake of CO is high, and to errors in inspired and expired CO analysis, particularly when the ratio of dead space to tidal volume is high. A summation of such errors presumably accounts for the very high value recorded for D_{CO} in Patient 2 when breathing CO in room air. In all other instances the correspondence between the two methods is as good as could be expected in view of known limits of analytical accuracy.

The topmost graph in Figure 3 is a plot of fractional CO uptake against D_{O_2}. Although there is a general tendency for fractional uptake to rise with increasing values of D_{O_2}, there is a considerable amount of scatter. This is probably due to the fact that fractional CO uptake decreases with increasing alveolar ventilation as well as decreasing with a lowered pulmonary diffusing capacity (5); in these studies the alveolar minute ventilation varied significantly. It is possible to calculate a value of D_{CO} from the fractional CO uptake if alveolar ventilation is known (16). This in effect corrects fractional CO uptake for variations in alveolar ventilation.

B. Critique of the measurement of D_{O_2}

The accuracy of D_{O_2} estimation by the Lilienthal-Riley technique has been discussed before (7). The method is inaccurate unless the end gradient, or mean alveolar to end capillary blood P_{O_2} difference is 6 mm. Hg or more. Since both exercise and 'alveolar-capillary block' cause this gradient to rise, the circumstances of the present studies were to the advantage of the method. If the gradients are sufficiently large, the estimate of D_{O_2} by this method appears to be good in health or in disease. The rate of combination of O_2 with intracorpuscular Hb, although faster than that for CO, interferes with the uptake of O_2 by the blood in the lungs in an analogous manner (17). The degree of underestimation of D_{O_2} because of this theoretical error is not at present known.

C. Critique of the measurement of D_{CO} steady state and breath holding

The presence of a CO tension in the capillary blood will interfere with the diffusion of CO into blood and if it is not allowed for, will produce an apparent underestimate of the true pulmonary diffusing capacity (17). There are two reasons for the existence of a significant capillary blood P_{CO}, the first of which is the presence in the blood of COHb in a large enough concentration to have a significant P_{CO} in equilibrium with it. Although the blood COHb was not analyzed in these studies, the patients were all non-smokers, so their ini-

tial mixed venous COHb would have been expected to lie between 0 and 5 per cent saturation (18). The effect of this COHb was ignored in both steady state and breath holding D_{CO} measurements. During the steady state procedure there was, in addition, a significant increase in mixed venous COHb, which can be calculated from the total CO uptake and an assumed blood volume. The values of alveolar P_{CO} used to calculate D_{CO} steady state in Table II have been corrected by subtracting from the actual alveolar P_{CO} the tension of CO in equilibrium with the average mixed venous COHb during the test at the mean capillary P_{O_2}. The calculation of D_{CO} breath holding needs no correction for blood COHb except in exceptional circumstances because: (a) the total amount of CO absorbed in the procedure is about a quarter of that taken up in the steady state D_{CO} measurement; (b) in addition, the alveolar P_{CO} is much higher; in the example of the calculation of breath holding D_{CO} of Patient 2 cited above, compare the alveolar CO concentration of 0.106 per cent initially, falling to 0.069 per cent at the end of 10 seconds, with the steady state alveolar CO concentration in Table II of 0.2 mm. Hg/713 or 0.028 per cent CO.

The second cause of a CO tension in the capillary blood during its passage through the alveolus is the slowness of the reaction of CO with intracellular Hb which prevents its being bound in the blood and allows it to pile up in solution in the plasma. This problem does in fact exist (17), but will result in an underestimate of D_{CO} by the same proportion in either the breath holding or steady state methods of estimating D_{CO}. The rate of combination of CO and Hb decreases as the blood O_2 tension rises. Therefore, measurements of D_{CO} by either breath holding or steady state methods must be done at a relatively constant alveolar P_{O_2} (actually constant capillary blood P_{O_2}) if the test is to be standardized. In the present studies all the breath holding tests were done under conditions which could reasonably be expected to produce an alveolar P_{O_2} of about 100 mm. Hg. On the other hand, the steady state D_{CO} measurements on each subject were made with two different alveolar P_{O_2} levels: 100 mm. Hg and 55 mm. Hg. At the lower O_2 tension, the rate of combination of CO and Hb should have been greater; as a result the capillary P_{CO} should have been less and the calculated steady state D_{CO} should have been greater. However, the calculated value of steady state D_{CO}, while breathing a low O_2 mixture, was greater than that when breathing air in only 4 out of 7 instances (Figure 3 and Table II). Since other data definitely show an increase in calculated D_{CO} by either steady state or breath holding techniques when the alveolar P_{O_2} is decreased (5, 9), the failure of the present measurements to show a similar change must probably be accounted for by the presence of analytical inaccuracies which conceal this effect. It must be recognized that the determinations of diffusing capacity reported in this paper put considerable strain on the analytical techniques which were available.

In the breath holding technique the calculation of the initial CO concentration in the expired alveolar gas depends upon the assumption that the inspired CO-He mixture is completely mixed with alveolar gas before significant absorption of CO into the blood takes place. The validity of this assumption is in turn based on (a) calculations of the rapidity with which diffusion mixes gases in the finer divisions of the respiratory tree (10), and (b) the fact that the expired alveolar CO concentration, when measured after various intervals of breath holding in normal subjects and plotted against time, back-extrapolates to the initial CO concentration as calculated on the basis of this assumption. Expired alveolar gas samples were obtained at various durations of breath holding in the present subjects (these data are not all included) and observation (b) is also true for them. Therefore, it appears valid to calculate the initial CO concentration in the collected expired alveolar sample from the dilution of the inspired He concentration, but the question may arise as to whether the sample derives from alveoli which have local diffusing capacities which are representative of the pulmonary diffusing capacity of the lung as a whole. D_{CO} breath holding (measured over a 10-second period) in normal subjects and in a limited number of patients with sarcoidosis and emphysema, varies less than 10 per cent when the expired alveolar sample analyzed is from the early or late parts of the expiration (15). Since the early parts of the expired alveolar gas come from different alveoli than the later parts (19), this suggests that the different parts of the expired

alveolar gas are reasonably representative in respect to the total pulmonary diffusing capacity.

The steady state D_{CO} method and D_{O_2} method for measuring pulmonary diffusing capacity are both subject to theoretical errors if pulmonary diffusing capacity is not distributed evenly throughout the lung in relation to alveolar ventilation (16), just as the breath holding D_{CO} estimate is subject to error if pulmonary diffusing capacity is not distributed throughout the lung evenly with respect to alveolar volume. In comparing D_{CO} breath holding with either D_{O_2} or D_{CO} steady state it must be borne in mind that conditions are not strictly comparable during the tests. The breathing maneuvers performed in the breath holding test may alter the state and number of the perfused capillaries and may do so to a different degree in the different subjects. Although the D_{CO} breath holding could not have been expected to be exactly equal to D_{CO} steady state or to D_{O_2}, particularly when the latter were measured during exercise, one of the purposes of this investigation was to determine whether it provides a convenient and reliable means of evaluating the state of the diffusing surface in patients. The results are sufficiently encouraging to warrant a more extensive investigation of the method.

SUMMARY AND CONCLUSIONS

Measurements of D_{CO} were made at rest on seven subjects by a modification of Krogh's breath holding technique. These findings were compared to simultaneous steady state measurements of D_{O_2} by the Lilienthal-Riley technique, of D_{CO} by the Filley technique and of fractional CO uptake as described by Bates. The latter methods were applied during exercise in five of the seven subjects. All but one of the subjects were hospital patients and together they provided a wide range of values for pulmonary diffusing capacity.

It is concluded for the present that the methods for the estimation of steady state D_{O_2} and D_{CO} provide similar values when allowance is made for the differences in physical characteristics between the two gases. Estimates obtained by the CO breath holding technique at rest show directional changes which are comparable to those obtained by the other methods during exercise, although the absolute values are lower. The fractional CO uptake, when uncorrected for changes in ventilation rate, is less reliable.

The optimum conditions for each method vary, and further studies of the advantages and limitations of each are required.

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PAPER ELECTROPHORESIS AS A QUANTITATIVE METHOD: MEASUREMENT OF ALPHA AND BETA LIPOPROTEIN CHOLESTEROL

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The development of methods for the separation and measurement of serum lipoproteins by ultracentrifugal flotation, fractional precipitation and zone electrophoresis has made possible studies of the metabolism and pathological significance of several lipoprotein fractions. Except for early studies by Lindgren, Elliott, Gofman, Jones, Lyon, and Strisower on the relationship of beta lipoprotein subfractions of certain flotation rates to atherosclerosis (1, 2) most of these studies have been concerned with alpha and beta lipoproteins, as defined by density in the ultracentrifugal techniques (3–5), by solubility in the chemical fractionation techniques (6) and by mobility in electrophoretic separation. Using a protein fractionation procedure developed by Cohn and his group (7), Barr, Russ, and Eder (8, 9) found that in 33 atherosclerotic individuals an average of only 13.6 per cent of the total serum cholesterol was associated with the alpha lipoprotein fraction as compared to 27.8 per cent in 85 normals.

In an attempt to avoid the complex equipment and technique and the large amount of serum required for the ultracentrifugal and chemical techniques, several workers have taken advantage of the ease of separation of alpha and beta lipoproteins by electrophoresis in anti-convection media such as starch and filter paper. The separated lipoproteins were then measured either by staining techniques, which are dependent on the solubility of lipid dyes in certain serum lipids (10–14 and others), or by the more specific method of elution and chemical determination of the cholesterol in the alpha and beta lipoprotein fractions (15–21). Thus Nikkilä (15), using 0.24 ml. of serum separated on three sheets of 11 × 30 cm. paper has shown a decrease in alpha lipoprotein cholesterol in atherosclerotic patients, and Kunkel and Slater (22) have made use of starch blocks as supporting media in order to avoid the "tailing" of lipoproteins encountered with filter paper. The techniques of cholesterol measurement used in these studies have the disadvantages of being time-consuming and of requiring larger amounts of serum than can be adequately separated on a single paper strip of reasonable size.

Zlatkis, Zak, and Boyle have recently described a method for the determination of the total cholesterol of whole serum without extraction, precipitation or hydrolysis, which is several times more sensitive than the Schoenheimer-Sperry technique and which gives equal color with cholesterol and cholesterol esters (23). Use of this method on whole serum has been criticized by Best, Van Loon, Wathem, and Seger (24) who found that it gave high values compared to the Schoenheimer-Sperry method, and by Fürst and Lange (25) who found variations in color with changes in temperature or amounts of reagent.

A method is described here for the determination of alpha and beta lipoprotein cholesterol on eluates from 0.02 ml. of electrophoretically separated serum. Cholesterol is determined by a modification of the Zlatkis, Zak, and Boyle procedure which gives satisfactory agreement with the Schoenheimer-Sperry technique. The small amount of serum required allows a clear cut separation of the alpha and beta fractions in a short migration distance without overloading of the paper and without measurable error due to "tailing." The method is suited for large scale clinical studies with regard to time, technique and apparatus involved and meets clinical standards of reproducibility and quantitative recovery.

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MATERIALS AND METHODS

Reagents

1. *Barbital buffer,* pH 8.6, 0.05 μ. 1.84 g. of diethylbarbituric acid and 10.3 g. of sodium barbital were dissolved in one liter of distilled water.

2. *Sudan black staining solution* was prepared according to Swahn (10). One liter of 60 per cent ethanol was saturated with 1 g. of Sudan black B (National Aniline Division, Allied Chemical and Dye Corporation) by bringing to the boiling point. The solution was cooled to room temperature, filtered, and stored in a closed container.

3. *Chloroform-methanol solvent:* Two volumes of redistilled chloroform were mixed with one volume of redistilled methanol just prior to use. These solvents were freshly distilled no more than two weeks before use.

4. *Standard cholesterol solution:* One hundred mg. of recrystallized cholesterol was dissolved in glacial acetic acid and made up to 100 ml. at 25° C. This solution was stored frozen in the refrigerator and warmed to 24° to 26° C. before samples were withdrawn. Under these conditions no measurable deterioration of the standard solution was found over a period of four months.

5. *Glacial acetic acid:* Except as otherwise specified, reagent grade glacial acetic acid (Fisher Chemical Co.) was used. Acetic acid was tested for the presence of glyoxylic acid which would give color with tryptophane by a modification of the method of Brice (26). Five hundredths ml. of 100 mg. per cent aqueous tryptophane was added to 0.95 ml. of the acetic acid to be tested. A volume of 0.65 ml. of sulfuric acid was then added, and the mixture was heated for 20 minutes at 56° C. A pink color indicates the presence of glyoxylic acid. Acetic acid which was used for cholesterol determinations gave a faint or negative reaction with this test.

6. *Ferric chloride-sulfuric acid cholesterol reagent* (23): One g. of reagent grade $FeCl_3 \cdot 6H_2O$ (Coleman & Bell Co.) was dissolved in glacial acetic acid and made up to 10.0 ml. in a volumetric flask. Five ml. of this solution was diluted to 500 ml. with concentrated sulfuric acid (Baker and Adamson C.P. reagent). The resulting pale yellow solution was stored in a separatory funnel plugged with a drying tube containing anhydrous calcium sulfate (Drierite) to prevent absorption of moisture from the air. Reagent kept in this way over one month gave the same results as fresh reagent.

Procedure

1. *Electrophoresis:* Electrophoretic separation was carried out on eight 2.9 × 30.0 cm. Whatman 3 MM filter paper strips in a cell of the hanging strip type (27). To minimize variations in the final color caused by material in the paper, all strips used in each determination were cut from the same sheet of filter paper and care was taken to keep the strips clean and free from perspiration. After the strips had been wet with buffer and allowed to drain for 15 minutes, 0.02 ml. of serum was applied in a streak at the apex of each strip with a micropipette (Microchemical Specialties Co., Berkeley, California), and a potential of 220 volts (14–17 m.a.) was applied for two hours. The strips were then spread flat to avoid shifts in the bands during drying, and dried with a current of air at room temperature. Each serum was run in duplicate along with an additional sample for staining.

2. *Staining:* Those strips which were to be used to locate the lipoprotein bands were dried horizontally for 10 minutes in an oven at 110° C., stained for one hour in Sudan black staining solution, rinsed for a few minutes in tap water, blotted, and again dried. One strip could be used for the location of the lipoprotein bands of two different sera by applying two 0.01 ml. samples to a strip with a 2 × 120 mm. slit cut down its center (Figure 1).

3. *Elution:* The apparatus used for eluting cholesterol from the strips is shown in Figure 2. A thermostatically controlled heating block maintains the solvent in the tubes at a steady boil with the vapors condensing on air cooled stainless steel condensers or "cold finger" condensers constructed from conical centrifuge tubes. Paper segments attached to the condensers are exposed to

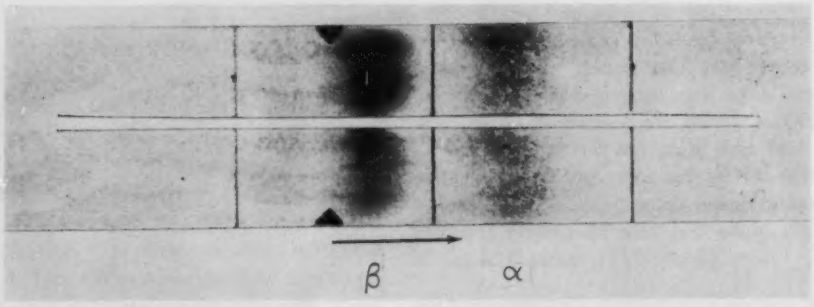

FIG. 1. SLOTTED PAPER STRIP SHOWING TYPICAL ALPHA AND BETA LIPOPROTEIN BANDS OF TWO SERA STAINED WITH SUDAN BLACK

The lines represent the point for division of the bands on corresponding unstained strips. The carets show the point of serum application.

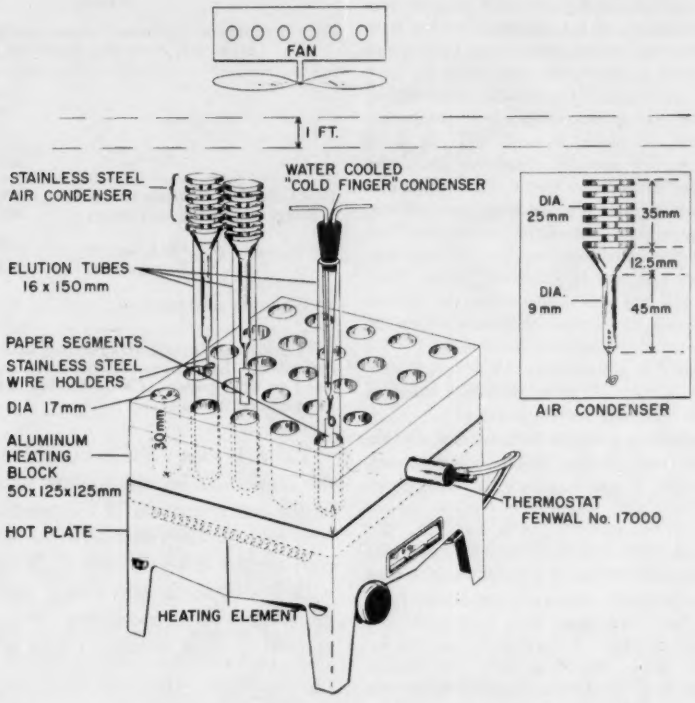

FIG. 2. APPARATUS USED FOR ELUTION OF SERUM CHOLESTEROL FROM PAPER STRIPS

a constant flow of fresh solvent, thus accomplishing an elution similar in principle to a Soxhlet extraction. The thermostat on the block is adjusted so as to maintain 4 ml. of water in an elution tube at 72 to 73° C. This provides for rapid boiling without excessive loss of solvent during the elution. The tops of the elution tubes are ground at a bevel to provide a close fit to the metal condensers.

The lipid patterns on the stained strips were marked off into two 3 cm. segments which best separated the alpha and beta fractions and which together included the entire lipid pattern (Figure 1). Identical segments were then marked off on the unstained strips by aligning them with the corresponding stained pattern at the point of serum application. These unstained segments were cut out, folded with tweezers in one direction to four or five thicknesses and forced into the wire loops on the condensers. A 3 cm. segment cut from the cathode side of a strip was used as a blank. Four ml. of chloroform-methanol solvent was added to each elution tube along with an alumina boiling chip, and the tubes were placed in the heating block and allowed to come to a boil.. The condensers were then placed in the tubes and adjusted so that the shafts did not touch the walls of the elution tubes, thus preventing the refluxing solvent from being drained off the condensers onto the walls of the tubes without passing through the paper segments. The segments were eluted for two hours and the tubes were allowed to stand overnight.

4. *Determination of cholesterol:* The cholesterol in the eluate was determined by a modification of the method of Zlatkis, Zak, and Boyle (23). After the condensers were removed from the tubes and a fresh boiling stone added, the solvent was again brought to a boil on the block. A cardboard cover with holes for the openings of the tubes was placed over the apparatus to help the evaporation by warming the tubes and preventing condensation of vapor on their walls. The cover was constructed so as to enclose the entire space above the heating block up to the top of the tubes. After the solvent had been allowed to evaporate for one hour the cover was removed, 1.00 ml. of glacial acetic acid was added to the dry residue in each tube, and the tubes were replaced in the heated block for 15 minutes to insure solution of the residue. At this point two standards were prepared by pipetting 0.02 ml. of the standard cholesterol solution into each of two elution tubes and adding 0.98 ml. of glacial acetic acid. A volume of 1.00 ml. of acetic acid was added to an elution tube for use as a reagent blank. After the tubes had cooled, 0.65 ml.

of ferric chloride-sulfuric acid cholesterol reagent was added to each tube from a 10 ml. burette, allowing it to run slowly down the wall of the tube from a point about 3 cm. above the level of the acetic acid so as to form a layer under the acetic acid. Immediately after adding the reagent each tube was struck sharply with the fingers to mix the reagents and placed in a 56° ± 1° C. water bath for 20 (17 to 23) minutes. Samples containing cholesterol developed a brownish purple color, while elution blanks were pale yellow. The solutions were allowed to cool to room temperature, transferred to matched 10 × 75 mm. round cuvettes, centrifuged briefly to remove bubbles, and read within one hour in a Coleman Junior Spectrophotometer at 560 mμ. In transferring the viscous samples to the cuvettes, the elution tubes were allowed to drain while supported in an inverted position by 1 × 110 mm. glass rods placed in the cuvettes. The optical density developed in the standard tubes showed a range of variation of 10 per cent over several months.

For the determination of eluted cholesterol by the Sperry and Webb modification of the Schoenheimer-Sperry procedure (28) it was necessary to combine eluates from two strips. The evaporation was carried out until about 1 ml. of solvent remained in each tube, and duplicate eluates were then combined, rinsing three times with 1 ml., 0.5 ml., and 0.5 ml. of chloroform-methanol. The combined eluates were evaporated to dryness, and the residue extracted three times with 1 ml., 0.75 ml., and 0.75 ml. of ethanol-ether (3:1), transferring the extracts to a 15 ml. conical centrifuge tube. Hydrolysis, digitonin precipitation and color development were then carried out in the usual manner.

RESULTS

Color development and elution

Determination of the total cholesterol of whole serum with the ferric chloride-sulfuric acid reagent without extraction, hydrolysis or precipitation (23) has in our hands given results which do not consistently agree with those obtained with the Schoenheimer-Sperry method, in confirmation of the report by Best, Van Loon, Wathem, and Seger (24). Use of acetic acid which had been refluxed over chromic acid and redistilled twice (29) did not improve the results.

However, when the reaction was carried out on chloroform-methanol extracts of serum which had been electrophoretically separated on paper strips, and the temperature during color development was controlled by use of a 56° water bath, satisfactory results were obtained.[8]

TABLE I

Color development with whole serum and eluted serum using acetic acid of varying degrees of purity *

		Net O.D. at 560 mμ		
		Acetic acid		
		Purified	Reagent	U.S.P.
H_2SO_4-$FeCl_3$ Reagent	Whole serum	.379	.407	.433
	Eluted serum	.366	.375	.360
H_2SO_4 only	Whole serum	.097	.126	.144
	Eluted serum	.068	.055	.062
Glyoxylic acid reaction		0	+	+++

* Purified acetic acid was refluxed over chromic acid and redistilled. Eluted samples are from serum streaked and dried on filter paper. Color development was carried out as described in the text.

Table I shows the influence of impurities in the acetic acid on the amount of color developed in the determination of cholesterol on whole serum and on chloroform-methanol extracts of serum streaks dried on paper. It may be seen that with lots of acetic acid giving increasingly strong glyoxylic acid reactions there is an increase in the amount of color developed with whole serum, but

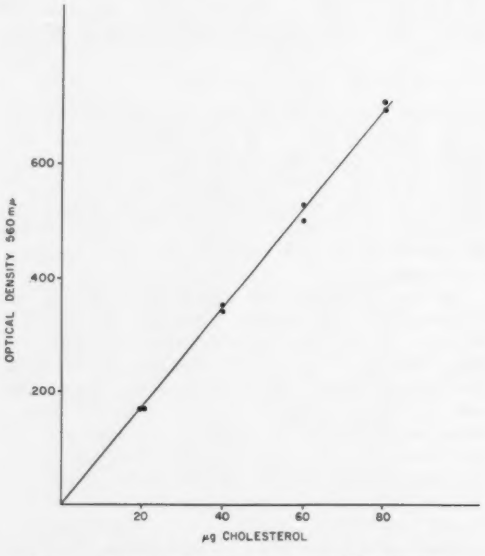

FIG. 3. STANDARD CURVE FOR PURE CHOLESTEROL

[8] After this work was completed, Zak, and his coworkers published a method for the determination of total serum cholesterol with the ferric chloride-sulfuric acid reagent on alcohol-acetone extracts of sera which shows satisfactory agreement with the Kingsley-Schaffert procedure (30).

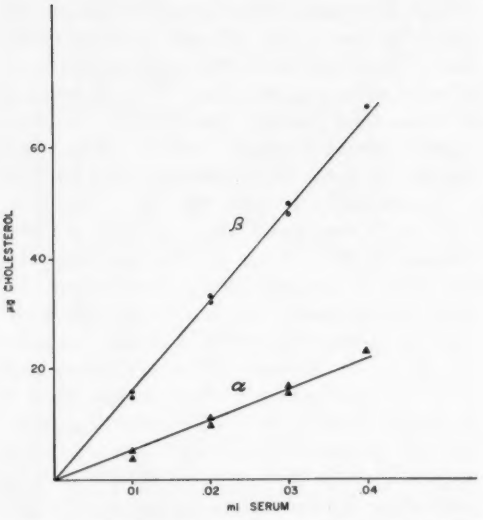

FIG. 4. DETERMINATION OF CHOLESTEROL IN ELUATES FROM ALPHA AND BETA LIPOPROTEIN FRACTIONS OF INCREASING AMOUNTS OF SERUM

that no such increase occurs with eluates of the same serum. If ferric chloride is omitted so that the reaction is carried out under the conditions of the glyoxylic acid-tryptophane reaction, the same results are obtained. The small amount of color developed in all samples without ferric chloride is probably due to the reaction of small amounts of oxidizing substances present in the reaction mixture with cholesterol.

Figure 3 shows that the color developed with increasing amounts of pure cholesterol increases linearly up to an optical density of at least 0.700 when measured in the Coleman Junior Spectrophotometer after 20 minutes color development in the 56° bath. After this incubation the color is stable for at least one hour at room temperature or for 20 minutes at 56°.

Figure 4 shows that a linear response is also obtained with increasing amounts of serum carried through the complete process of electrophoretic separation, elution, and color development. The total cholesterol of this serum as determined by the Schoenheimer-Sperry technique was 212 mg. per cent, as compared to 216 mg. per cent obtained by adding the alpha and beta values shown. Thus the efficiency of separation and elution of the serum is not dependent on the quantity of material involved over a range of one-half to two times the amount ordinarily used.

TABLE II

Recovery of added cholesterol

Serum	Fraction	Serum cholesterol μg.	Found after addition of 20 μg. cholesterol	Recovery of added cholesterol μg.	Recovery %
Pool I*	Alpha	8.9	29.6	20.7	104
	Beta	31.5	50.2	18.7	94
1149	Alpha	7.1	27.6	20.5	103
	Beta	36.2	57.0	20.8	104
1221	Alpha	10.2	31.2	21.0	105
	Beta	23.0	43.5	20.5	103
1579	Alpha	8.5	29.3	20.8	104
	Beta	41.4	62.0	20.6	103

* Pooled serum from 10 blood donors.

Table II shows the recovery of added cholesterol, which was streaked over alpha and beta fractions of sera which had been previously separated and dried. The quantitative recovery obtained is evidence against the presence in the serum of inhibitors or other impurities affecting the color development due to cholesterol.

A comparison of the results obtained with the complete separation, elution and color development procedure on six different normal and abnormal sera with the results of Schoenheimer-Sperry determinations of total serum cholesterol on the same sera is shown in Table III. The sum of the values obtained for alpha and beta lipoprotein cholesterol agrees with the results of the Schoenheimer-Sperry method within the sum of the er-

TABLE III

Recovery of eluted cholesterol determined by the ferric chloride and Schoenheimer-Sperry methods

Serum	Schoenheimer-Sperry serum cholesterol mg. %	Ferric chloride eluted cholesterol (sum of alpha and beta) mg. %	Recovery %	Schoenheimer-Sperry eluted cholesterol mg. %	Recovery %
1589	202	192	95	213	106
1590	331	326	99	322	97
1592	238	258	110	247	103
1593	332	337	102	309	96
1595	328	330	101	306	93
Pool II*	288	261	91	264	92
Average recovery			100		98

* Pool of 5 sera.

TABLE IV

Elution of cholesterol from alpha and beta lipoproteins

Elution period	μg. Cholesterol recovered	
	Alpha	Beta
0 to 1½ hrs.	10.3	34.4
1½ hrs. to 2 hrs.	−0.1*	−0.4*
2 hrs. to 4 hrs.	−0.2*	0.4
4 hrs. to 19 hrs.	−0.2*	0.3

Serum: Pool I from 10 blood donors.
Cholesterol applied (Schoenheimer-Sperry method): *42.4* μg.
Cholesterol recovered (Ferric chloride method, sum of 1½ hr. alpha and beta values): *44.7* μg.

* Negative figures represent values lower than the blanks. These differences are no greater than the error of the determination.

rors of the two methods. To insure that the apparently complete recovery was not an artifact due to a combination of incomplete elution and erroneously high values with the ferric chloride procedure, the same sera were electrophoretically separated, the entire lipid containing area was eluted, and the combined eluates from two strips were analyzed for cholesterol by the Schoenheimer-Sperry procedure. The last two columns of Table III show that these values agree satisfactorily with those obtained with the ferric chloride method.

As a further test of the completeness of the two-hour elution procedure, the alpha and beta segments of two strips were eluted for a total of nineteen hours, transferring the segments to fresh elution tubes at intervals and determining the cholesterol extracted during each period. As shown in Table IV, no additional cholesterol was recovered after one and one-half hours of elution. The recovery (sum of one and one-half hours alpha and beta values *vs.* Schoenheimer-Sperry total) was 105 per cent.

The blank values obtained by elution of an area of the paper strip containing no protein could be lowered somewhat by extraction of the strip with fat solvents before use. However, since the net values for eluted serum cholesterol were not changed by such a procedure, it was not ordinarily carried out. The buffer salts in the dried paper, which are eluted along with the lipid material, do not give color or affect the color reaction.

Electrophoretic separation

Separation of the alpha and beta lipoprotein fractions of serum was carried out simultaneously on eight filter paper strips moistened with pH 8.6 barbital buffer in the cell previously described (*27*). Using 0.02 ml. of serum, a clear cut separation of alpha and beta lipoproteins as revealed by Sudan black staining was invariably obtained. Figure 1 shows a typical "marker" strip which was used to locate the lipoprotein bands for each of the sera run in the same cell.

The point used for dividing the alpha and beta fractions was about 2 mm. ahead of the sharply defined front of the beta lipoprotein band; no evidence was obtained by staining for the presence of other lipoprotein fractions in practicably measurable amounts. However, in certain pathological sera, the "beta lipoprotein" band moved ahead of the beta globulins as revealed by protein staining. Since all gradations of mobility between $alpha_2$ and beta globulin were observed for this lipoprotein fraction in a series of such sera, while the mobility of alpha lipoproteins was remarkably constant, it seemed most reasonable to consider these to be abnormal forms of beta lipoprotein rather than another fraction of alpha lipoprotein. No attempt was made to distinguish between chylomicrons and beta lipoprotein adsorbed at the application point; such a differentiation would presumably be of little importance in any case because of the low cholesterol content of chylomicrons (1).

Under these conditions of electrophoretic separation, the leading edge of the alpha lipoprotein band migrated 3 to 4 cm. from the origin. This relatively short migration distance was selected in order to minimize the possibility of error due to "tailing" of alpha lipoprotein and the high blank values obtained if large amounts of paper are eluted. As mentioned above, "tailing" of beta lipoproteins is of no consequence, since any beta "tail" is eluted with the band itself. The linear recovery of cholesterol obtained with electrophoresis and elution of increasing amounts of serum (Figure 4) rules out the possibility of significant adsorption of the alpha lipoprotein of the kind ordinarily observed, in which a fairly constant amount of material is adsorbed on a given area of paper regardless of the amount applied. The occurrence of an appreciable amount of this type of "tailing" would result in a displacement of the alpha and beta lipoprotein lines so that the latter would intersect the ordinate above zero, and the former below zero, as found with albumin "tail-

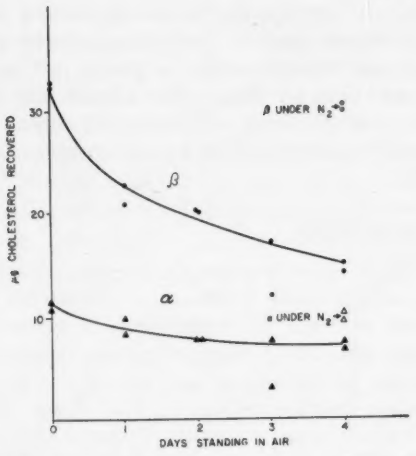

FIG. 5. DECOMPOSITION OF ALPHA AND BETA LIPOPROTEIN CHOLESTEROL ON PAPER STRIPS STANDING IN AIR AT ROOM TEMPERATURE

ing" (31, 32). It is likely that albumin, passing over the paper surface in front of the alpha lipoprotein, covers up the available adsorptive sites on the paper, thus "laying down a carpet" for the globulin fractions.

Decomposition of cholesterol

The recovery of cholesterol from a series of identical strips eluted at varying periods of time after electrophoretic separation is shown in Figure 5. It may be seen that after the strips had stood in air at room temperature for four days there was a 55 per cent loss of cholesterol as determined by the ferric chloride procedure. Strips which had been kept under nitrogen for the same period of time showed only a 16 per cent loss, suggesting that oxidation was responsible for the decomposition. In a similar experiment strips which had been allowed to stand for seven days in air showed a 42 per cent loss by the ferric chloride method, and a 27 per cent loss by the Schoenheimer-Sperry procedure. In order to avoid this type of decomposition, the dried strips were eluted within a few hours of the electrophoretic separation. No losses were observed on allowing the eluted cholesterol to stand in solution overnight.

Oxidation of cholesterol under these conditions is not unexpected in view of the results of Bergström and Wintersteiner (33) who observed a rapid oxidation of colloidal cholesterol in water by a stream of air at elevated temperatures and a significant rate of decomposition at 37°.

In view of these observations, it appeared necessary to examine the possibility of losses of cholesterol during the electrophoretic procedure itself. Experiments in which pure cholesterol was applied to strips from a solution of acetic acid, dried, the strips moistened with buffer, and the complete electrophoretic and elution procedure carried out, did show recoveries which were 12 to 15 per cent low. However, numerous experiments on serum, including those reported in Table III, failed to show any evidence of decomposition and no difference in cholesterol recovery was observed if the electrophoretic procedure was carried out at 0 to 1° C. in an attempt to decrease any oxidation that might be taking place by lowering the temperature. Table II, already referred to, shows that pure cholesterol added to the area of the alpha and beta bands is recovered completely. It appears that decomposition of cholesterol does not readily take place in solution, but is appreciable when exposed to air in the solid or colloidal state. It is possible that serum exerts a specific protective effect because of the presence of antioxidants in serum. It is generally known that while lipoproteins are stable in serum for several weeks at 5°, they undergo rapid oxidation in the absence of serum as shown by increases in electrophoretic mobility and decreases in ultracentrifugal flotation rates (34, 35).

As a further check on the method, the ratios of the alpha and beta lipoproteins of three pooled sera were determined by both the Schoenheimer-

TABLE V

Comparison of the Schoenheimer-Sperry and ferric chloride methods in the determination of lipoprotein cholesterol distribution

Serum	% of total cholesterol in alpha lipoprotein fraction	
	Schoenheimer-Sperry method	Ferric chloride method
Pool III*	23.4	22.9
	21.4	21.9
Pool IV*	20.5	19.7
	19.4	19.4
Pool V*	20.4	19.9
	18.3	18.5

* Pooled serum from 10 blood donors.

TABLE VI

Reproducibility of lipoprotein cholesterol distribution and total cholesterol in five separate determinations on six normal and abnormal sera

Serum	Experiment No.	% Alpha lipoprotein cholesterol	Total cholesterol (sum of alpha and beta) *mg. %*
1149	1	19.0	240
	2	18.6	218
	3	17.8	224
	4	17.3	230
	5	19.6	235
	Average	18.5	229
1209	1	16.0	315
	2	15.7	303
	3	16.8	307
	4	16.6	327
	5	16.7	322
	Average	16.4	315
1221	1	30.4	191
	2	30.7	170
	3	31.6	175
	4	32.5	191
	5	31.8	197
	Average	31.4	185
1493	1	14.1	336
	2	13.8	312
	3	13.6	318
	4	14.3	334
	5	15.4	333
	Average	14.2	327
1579	1	17.5	280
	2	16.9	252
	3	18.6	269
	4	16.2	274
	5	17.9	283
	Average	17.4	272
Pool I*	1	24.7	228
	2	22.0	211
	3	24.4	218
	4	25.0	222
	5	26.9	230
	Average	24.6	222

* Pooled serum from 10 blood donors.

Sperry and ferric chloride methods. Because of the comparatively low sensitivity of the Schoenheimer-Sperry method, it was necessary to elute segments from strips on which 0.04 ml. of serum had been run, and to combine the eluates from four alpha segments and from two beta segments for the determination of cholesterol in the respective fractions. The results of such an experiment, shown in Table V, indicate that closely comparable values for the distribution of cholesterol between the alpha and beta lipoproteins are obtained by both methods and that no combination of errors in the ferric chloride method is giving rise to incorrect values for alpha and beta lipoprotein cholesterol while giving satisfactory agreement between the two methods for total cholesterol (Table III).

Reproducibility

The results of a study of the reproducibility of the method under conditions of routine use are shown in Table VI. The complete separation, elution and color development procedure was carried out in duplicate on each of six sera in five separate experiments over a period of nine days. The widest range of variation observed was from 22.0 to 26.9 per cent for the fraction of total cholesterol found in alpha lipoprotein (24.4 to 26.9 per cent if one low value obtained for this serum is omitted), and from 252 to 283 mg. per cent for the total cholesterol (sum of alpha and beta fractions).

DISCUSSION

The available methods for determination of lipoprotein concentration are dependent on refractive index increment, dye binding capacity, or the content of some chemically defined material such as phospholipid or cholesterol in the lipoprotein molecule. Of these methods, the determination of cholesterol has the advantage of being based on a specific chemical reaction of a substance of known importance in atherosclerosis, while the refractive index increment is dependent on the concentration of both protein and lipids. The measurement of uptake of lipid dyes by lipoprotein fractions, which is dependent on solubility interrelationships between dye, solvent and lipid, does not appear to have as firm a basis as either of these methods (36). It should be kept in mind that lipoprotein concentrations expressed in these various units are not directly comparable because of the different composition of different normal and abnormal lipoproteins.

The wide application of some of the methods of serum lipoprotein separation and estimation has been restricted by the time, technique, amount of serum and equipment required. The estimation of alpha and beta lipoprotein cholesterol on

eluates from electrophoretically separated serum has the advantages of being relatively simple and of requiring a minimum of serum and equipment. The time required and the number of sera which can be conveniently analyzed together are approximately the same as for the Schoenheimer-Sperry determination of total cholesterol, while both total cholesterol and its lipoprotein distribution are determined with the electrophoretic procedure.

SUMMARY

1. A method is described for the determination of the alpha and beta lipoprotein cholesterol of 0.02 ml. of serum separated by electrophoresis on filter paper.

2. Under the conditions described, the following criteria are satisfied:

a. A linear increase in color for increasing amounts of pure cholesterol and eluted alpha and beta lipoprotein cholesterol.

b. Agreement, within the errors of the two methods, of total cholesterol and of per cent alpha lipoprotein cholesterol as determined by the method described and by the Sperry and Webb modification of the Schoenheimer-Sperry procedure.

c. Complete recovery of eluted lipoprotein cholesterol.

d. Clear cut separation of alpha and beta lipoproteins.

e. Satisfactory reproducibility of the results in repeated determinations on the same sera.

3. Dry cholesterol on filter paper strips exposed to air at room temperature undergoes decomposition.

4. The method is comparable, in terms of time and number of sera analyzed, to the Schoenheimer-Sperry technique.

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PAPER ELECTROPHORESIS AS A QUANTITATIVE METHOD: THE STAINING OF SERUM LIPOPROTEINS

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Electrophoresis of serum on filter paper followed by staining [3] of the lipoprotein fractions with lipid dyes is technically the simplest method for the separation and estimation of serum lipoproteins. A number of procedures for the staining of separated lipoproteins on paper with various lipid dyes have appeared (1–8), but the only comprehensive consideration of the quantitative aspects of dye binding by serum lipids on paper is the very careful study reported by Swahn (9), which was primarily concerned with the quantitative aspects of the staining of whole serum on paper with a preparation of the lipid dye Sudan black.

A critical study of some of the characteristics of the staining of electrophoretically separated lipoproteins on paper and a detailed procedure for a semi-quantitative empirical determination of serum lipoprotein distribution with the lipid dye oil red O are reported here.

MATERIALS AND METHODS

Electrophoretic separation of serum lipoprotein fractions was carried out on eight 2.9 × 30.0 cm. Whatman 3 MM filter paper strips moistened with 0.05 μ pH 8.6 diethyl barbiturate buffer in the cell previously described (10). A volume of 0.02 ml. of serum was applied with a micropipette to the apex of each paper strip, which previously had been thoroughly wet with buffer and allowed to drain in the closed cell for 15 minutes. A constant potential of 220 volts (about 14–17 m.a.) was applied for two hours, after which the strips were spread flat, pulled almost taut and dried in an oven at 110 to 120° C. for 15 minutes. No significant differences in lipid dye uptake or distribution were found when the period of oven drying was varied from 10 to 40 minutes.

Oil red O staining solution was prepared by adding 0.4 g. of solid dye (lot 15123, National Aniline Division, Allied Chemical and Dye Corp.) to a liter of 60 per cent ethanol (V/V), bringing the mixture to a boil in a flask fitted with a condenser to prevent loss of alcohol, and allowing the solution to cool overnight in a 30° C. incubator with constant stirring by means of a magnetic stirrer. Omission of stirring resulted in the formation of a fine suspension of dye which was difficult to clarify. The solution (at about 33°) was filtered and kept in the incubator. When the same solution of dye was used for the staining of five groups of eight strips each over an eight-day period, the total amount of dye taken up by a given serum sample decreased by 25 per cent, but the relative amounts of dye bound by the alpha and beta lipoprotein fractions was the same at the end of the period as at the beginning.

Staining of strips was carried out for 18 hours at 30° C. The strips were held in racks which were placed in 1 liter of staining solution in a closed container ("plastic sandwich boxes," American Thermos Bottle Co.). After removal from the staining solution the rack was immediately placed in a container filled with tap water at room temperature and rinsed with constant agitation until the alcohol was removed from the paper (about two minutes). The strips were then blotted and allowed to dry at room temperature. Although some fading of dye takes place on prolonged exposure of the dry strips to bright light, no significant difference in dye content was found between strips eluted immediately after staining and strips which had stood for a month without exposure to bright light.

Staining with Sudan black B (NZb 6; National Aniline Division, Allied Chemical and Dye Corp.) was carried out according to the directions of Swahn (9). Because of a tendency of this dye to develop a brownish discoloration on standing, Sudan black strips were eluted shortly after the staining procedure.

Elution of dye was carried out with a solution containing 125 ml. of glacial acetic acid diluted to 500 ml. with absolute ethanol (9). The stained strips were marked off into two 2.5 cm. segments containing the alpha and beta lipoprotein bands, with the point of division of the two segments about 2 mm. ahead of the sharply defined front of the beta band. Five cm. segments were marked off from the protein free cathode side of the

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[3] The use of the terms "stain" and "dye" is not meant to imply the existence of a chemical union between "dye" and lipid.

strips to serve as blanks. The segments were cut out, placed in 5.0 ml. of acetic acid-ethanol in stoppered tubes, and shaken at 250 1.5 inch strokes per minute for two hours at room temperature. Elution was complete after this period of shaking as shown by the absence of any change in readings after a further two hours of shaking. The solutions were read with a Beckman model DU spectrophotometer at 520 mμ, which is at or close to the rather broad absorption maxima of all of the colored components in oil red O preparations. The concentration of oil red O has a linear relationship to optical density up to a reading of 1.3 in the Beckman instrument. Sudan black gives a linear response up to an optical density of 0.7 at 590 mμ in the Coleman Junior spectrophotometer, and was measured in this instrument. The readings obtained on eluates from the 5 cm. blank segments, were divided in half to correspond to the 2.5 cm. segments, and were then subtracted from the values obtained for the alpha and beta bands. Optical densities obtained for the alpha lipoprotein segments with oil red O were about double the blank values for most sera; the beta lipoprotein values were correspondingly higher. The data reported in this paper are in terms of optical density of the 5 ml. elution samples at 520 mμ after subtraction of the blank values unless otherwise specified; no attempt was made to convert the results to actual quantities of dye bound because of the heterogeneity of the dye preparations.

Direct optical scanning of dry strips was carried out with the instrument previously described (11), using a 2×23 mm. slit and a 525 mμ Bausch and Lomb first order interference filter with a tungsten light source. Paper was not "cleared" with mineral oil (12) or glycerol (13) because of the solubility of lipid dyes in the former and the appearance of artifacts due to difficulty in wetting protein bands with the latter. Because of the considerable background variation with consequent difficulties in selecting a true base line, the non-linearity of the relationship between dye concentration and optical density on paper (12, 14, 15) and the ease of eluting dye from only two lipoprotein fractions, quantitative measurements were not attempted with the direct scanning procedure.

RESULTS

Electrophoretic separation

The separation of alpha and beta lipoprotein fractions by electrophoresis on filter paper under the conditions described has been discussed elsewhere (16). A clear separation of alpha and beta

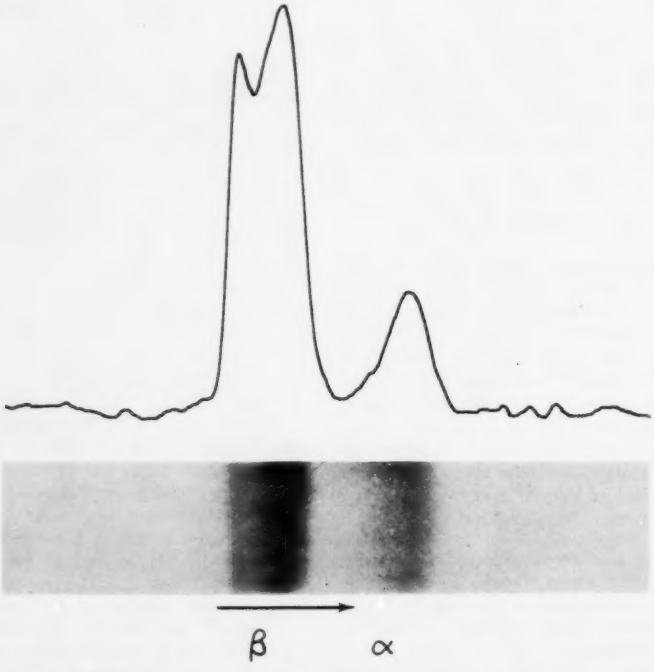

FIG. 1. ELECTROPHORETICALLY SEPARATED NORMAL SERUM ON PAPER STAINED WITH OIL RED O AND A DIRECT PHOTOELECTRIC SCAN PATTERN OF THE SAME STRIP

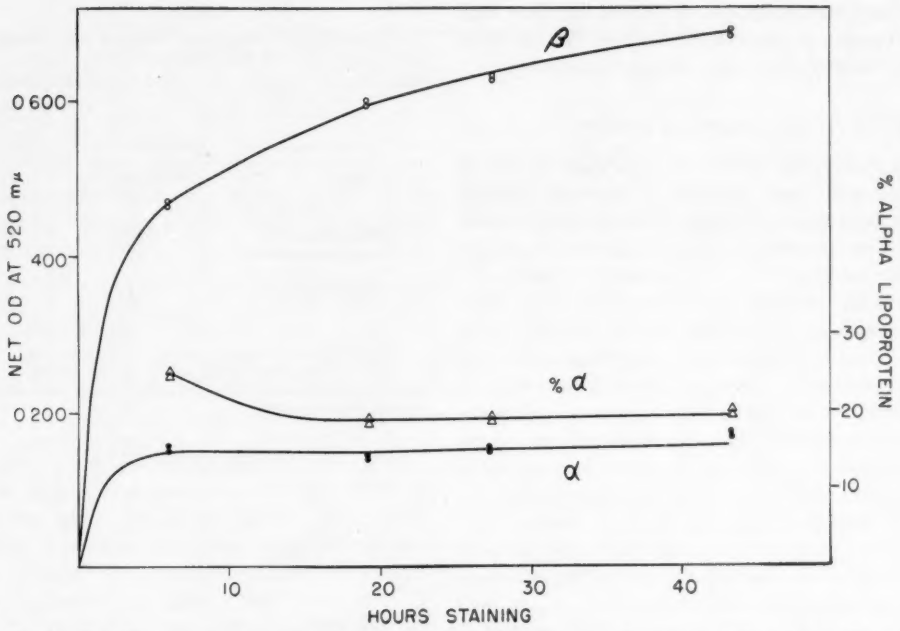

FIG. 2. DYE UPTAKE OF ALPHA AND BETA LIPOPROTEIN FRACTIONS OF SERUM WITH INCREASING TIME IN THE STAINING SOLUTION

bands is uniformly obtained, and except for chylomicrons and beta lipoprotein adsorbed at the origin and certain abnormal "beta lipoproteins" of increased mobility, no evidence has been obtained for the presence of subfractions in practicably measurable amounts. The occurrence of significant amounts of "tailing" of alpha lipoprotein is unlikely in view of the linear dye uptake obtained on separation and staining of increasing amounts of this fraction (Figure 3). A typical stained strip obtained with normal serum, and a tracing of a direct optical scan pattern of the same strip are shown in Figure 1.

Staining

Figure 2 shows the relationship between the duration of staining and the amount of oil red O taken up by electrophoretically separated alpha and beta lipoprotein fractions from a saturated solution of dye in 60 per cent ethanol. Although some dye uptake continues up to at least 40 hours, most of the dye is taken up during the first 18-hour period, the time ordinarily used for staining, and there is little or no change in the distribution of dye between the alpha and beta fractions after this time. Similar studies of the uptake of oil red O from 55 per cent and 50 per cent ethanol and of Sudan black from 40 per cent ethanol have indicated that equilibrium is approached somewhat more slowly with these solvents.

The relationship between lipoprotein concentration and amount of dye bound is shown in Figure 3. A volume of 0.01 to 0.04 ml. aliquots of serum were electrophoretically separated and stained, and the dye was eluted from the alpha and beta lipoprotein fractions. Although the uptake of dye by alpha lipoprotein is linear with increasing lipoprotein concentration, there is a deviation from linearity with the more concentrated beta lipoprotein. As a result of this deviation the per cent of the total dye bound by the alpha lipoprotein fraction increased from 20.8 per cent at the lowest concentration to 23.7 per cent at the highest concentration of lipoproteins studied in this experiment. Since part of the alpha lipoprotein fraction, which shows no such deviation, is in the same area of the paper strip as the very concentrated albumin fraction, it appears unlikely

that high concentrations of protein interfere with dye uptake or are responsible for the deviation from linearity observed with beta lipoprotein.

Effect of the dye solvent on staining

In theory, the uptake of lipid dyes by fats is supposed to be a process of physical solution, with the greater solubility of the dye in lipid than in solvent resulting in a high concentration of dye in the lipid (17, 18). A quantitative interpretation of this picture requires the existence of a two phase system of lipid and solvent between which dye would be distributed at equilibrium according to a distribution coefficient defined by the relative solubility of the dye in the two phases. Since the change in dye concentration in the solvent during the staining procedure is negligible because of the large volume of solution, the concentration of dye in the lipid should reach a constant value at equilibrium. Provided that none of the lipid phase is dissolved in the dye solvent during the equilibration period, the amount of dye found in the lipid phase would then be a measure of the amount of lipid present.

If this picture is correct, it follows that reexposure of stained lipid to solvent without dye should result in the removal of dye from the lipid until a new equilibrium is set up. Also, since the amount of dye taken up by a given amount of lipid should be directly proportional to the concentration of dye in the solvent, there should be little variation in the total amount of dye taken up by a given amount of lipid when stained in different solutions of dye at approximately the same concentration.

Several lines of evidence indicate that this hypothesis cannot be applied without reservations to the staining of lipid on paper by lipid dyes. The heterogeneity of both lipids and dyes and the large variations in dye uptake obtained for the same amount of lipid with dye solutions of almost identical concentration are described below; an experiment to study the solubility of lipid in the dye solvent is described here.

Strips on which 0.02 ml. of serum had been separated were allowed to stand for 18 hours in 60 per cent ethanol at room temperature and at 30° C. and were then rinsed in water, dried, and stained in a saturated solution of dye in 60 per cent ethanol. Identical control strips were carried through the same procedure except for the preliminary soaking in alcohol. The uptake of dye by the alpha and beta lipoprotein fractions on strips treated in this manner is shown in Table I. The total dye uptake is reduced 25 to 35 per cent by the preliminary soaking, and this decrease is greater for the alpha than for the beta fraction, thus giving rise to a different distribution of dye between the two fractions. In similar experiments a 27 per cent decrease in dye uptake was found after preliminary washing in diethylene glycol and a 22 per cent decrease after 50 per cent ethanol (in the latter case followed by staining in 50 per cent rather than 60 per cent ethanol).

TABLE I

Solubility of serum lipids in 60 per cent ethanol in the absence of dye *

Pre-treatment	Net total dye uptake (*O.D. 520 mμ/5 ml.*)	Alpha lipoprotein dye uptake (*% of total*)
Control	0.803	22.2
	0.791	21.1
60% ethanol at room temperature	0.570	16.1
	0.590	15.1
60% ethanol at 30° C.	0.521	12.9
	0.532	14.1

* Identical strips were placed in 60 per cent ethanol at room temperature or at 30° C. for 18 hours, rinsed in water, and stained. Control strips were not placed in ethanol until stained.

Although this type of experiment provides no information as to the solubility of serum lipids in 60 per cent alcohol that is saturated with dye, it does indicate that some of the lipids are soluble in the dye solvent in the absence of dye, but are not dissolved in the presence of dye. The losses obtained with the preliminary washing cannot be due simply to an increased length of exposure to 60 per cent ethanol as compared to the control strips, since prolonged exposure to the same solvent in the presence of dye results in an increase rather than a decrease in dye uptake (Figure 2).

In an attempt to find a staining system in which lipid would not be dissolved by the solvent, several aqueous media were tested in which dye was held in solution or suspension by gelatin (19), gum arabic, saponin, polyoxyethylene sorbitan

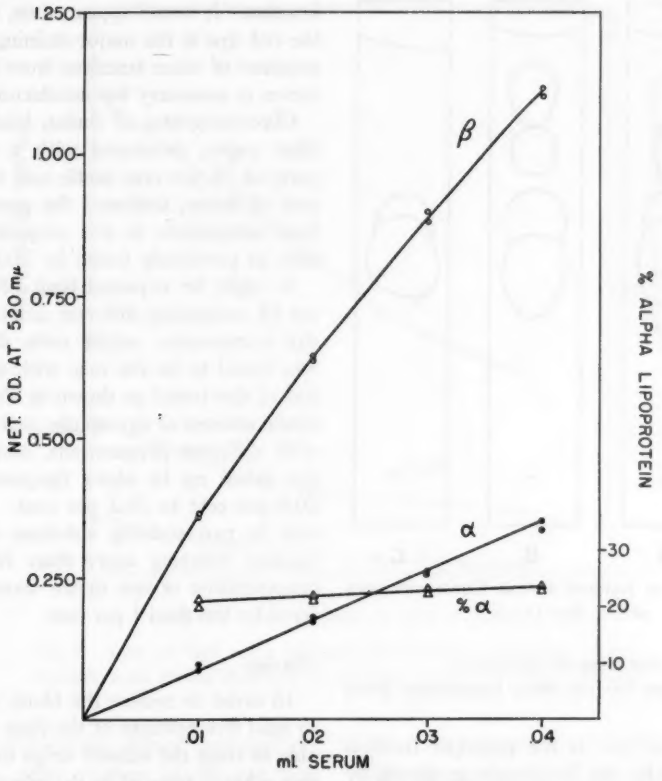

FIG. 3. DYE UPTAKE OF INCREASING AMOUNTS OF ELECTROPHORETICALLY SEPARATED SERUM STAINED WITH OIL RED O

monolaurate (Tween 20 [4]) or polyoxyethylene lauryl alcohol (Brij 35 [4]). Many preparations of this type caused artifacts by precipitation of dye on protein fractions which did not contain lipid. Saturated solutions of oil red O in acetone added to an equal volume of 1 per cent gelatin in 1 per cent acetic acid and allowed to stand at 37° overnight (19) produced good lipid stains, but led to precipitation of dye on the paper resulting in too high a background for practical use. Clear stable solutions of oil red O could be prepared in 0.1 per cent Tween 20 or Brij 35 by adding 0.1 to 1.0 ml. of a saturated solution of dye in dioxane to 100 ml. of solution; fairly good stains could be obtained from these preparations at 57°, especially with Brij, but it was found that aqueous solutions of these detergents have the same disadvantage as 60 per cent ethanol in that they wash out a fraction of the serum lipids from the paper strip.

Composition of the staining solution

Commercially available oil red O consists of at least four chromatographically distinct colored components. In Figure 4 are shown tracings of reversed phase chromatograms of the crude dye, the dye present in the staining solution and the dye which actually stains lipid (obtained by elution of a stained lipoprotein band). The separations were obtained in 16 hours at room temperature with Whatman 3 MM paper which had been dipped into a 30 per cent solution of mineral oil in ether and allowed to dry, using water saturated n-butanol as developing solvent. The principal component in each case is the slowest mov-

[4] We wish to thank the Atlas Powder Company for samples of these materials.

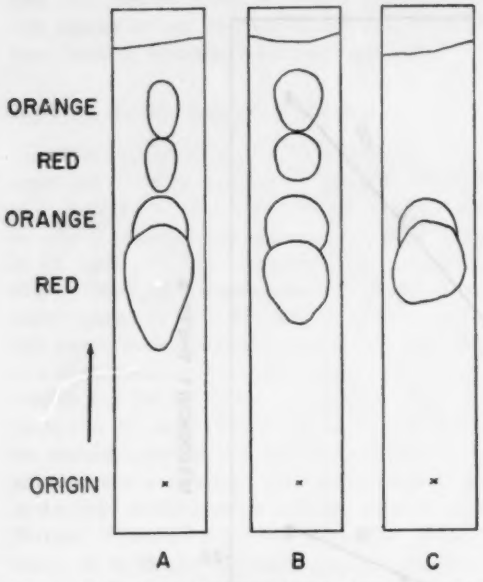

FIG. 4. TRACINGS OF REVERSE PHASE CHROMATOGRAMS OF OIL RED O

A. CRUDE DYE
B. DYE FROM SOLUTION USED FOR STAINING
C. DYE ELUTED FROM STAINED BETA LIPOPROTEIN BAND

ing red band, and this is the principal fraction which actually stains the lipoprotein as shown by chromatography of the eluted material. A slightly faster moving orange component is present in greater concentration in the staining solution than in the original dye preparation because of the use of a large excess of solid dye for saturation of the staining solution.

Attempts to stain lipoproteins with purified preparations containing less of the orange component were uniformly unsuccessful. When chromatographically homogeneous preparations of the main red component, prepared by fractional precipitation from dioxane-water or by chromatography on alumina, were used for staining only very dilute dye solutions and faint stains were obtained; the same results were obtained with staining solutions which were saturated with a minimum amount of the solid dye so that they contained little of the minor components. No success was achieved with attempts to substitute non-ionic detergents for the orange component, which might be acting by solubilizing or stabilizing the red fraction. It would appear, then, that even though the red dye is the major staining component, the presence of other fractions from the crude preparation is necessary for satisfactory staining.

Chromatograms of Sudan black B on circular filter paper, developed with a mixture of two parts of 25 per cent acetic acid in ethanol to one part of water, indicated the presence of at least four components in this preparation of this dye also, as previously found by McDonald (20).

It might be expected that different lots of oil red O, containing different amounts of the minor dye components, might stain differently. This was found to be the case with the four different lots of dye tested as shown in Table II. The absolute amount of dye uptake varied up to two-fold with different preparations, and the fraction of dye taken up by alpha lipoprotein varied from 20.8 per cent to 26.3 per cent. It is of interest that in two staining solutions which gave dye uptakes differing more than 100 per cent, the concentration of dye in the staining solution differed by less than 1 per cent.

Rinsing

In order to reduce the blank value due to dye in lipid free portions of the filter paper it is desirable to rinse the stained strips to remove residual dye solution trapped in the paper fibers; if a concentrated dye solution is used for staining, rinsing becomes absolutely necessary if a reasonable pattern to background ratio is to be achieved. A necessary requirement of any rinsing procedure, however, is that it remove only background dye, not any of the dye bound to lipoprotein.

TABLE II

Oil red O uptake of separated lipoprotein fractions using different lots of dye

Lot number	Concentration of staining solution (*O.D. 520 mμ*)	Net total dye uptake by lipoprotein (*O.D. 520 mμ/5 ml.*)	Alpha lipoprotein dye uptake (*% of total*)
12670	3.108	1.068	24.3
		1.063	24.6
14714	3.432	1.114	22.4
		1.133	22.3
15123	3.408	0.630	21.9
		0.586	20.8
15448	3.402	1.354	26.3
		1.416	26.3

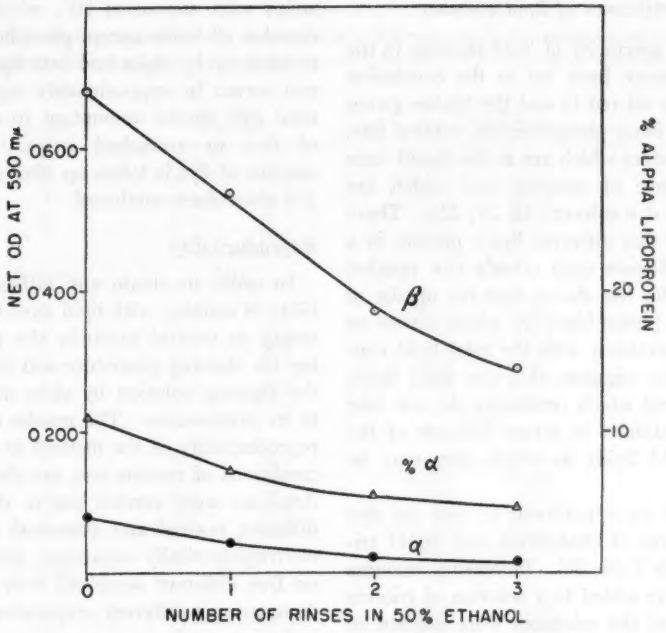

FIG. 5. LOSS OF DYE FROM ELECTROPHORETICALLY SEPARATED SERUM STAINED WITH SUDAN BLACK B ON RINSING IN 50 PER CENT ETHANOL

The recovery of dye from stained separated serum lipoproteins after rinsing in 50 per cent ethanol is shown in Figure 5. Serum was electrophoretically separated on Whatman No. 1 paper strips, stained with Sudan black B, and eluted after one, two, and three 15-minute rinses as described by Swahn (9). Corresponding background values were subtracted from each lipoprotein value. There is a considerable loss of dye in excess of background from both lipoprotein fractions, and the proportionally greater loss from the less concentrated alpha fraction results in progressively lower values for the portion of the total dye bound by this fraction.

Loss of dye from stained lipoprotein fractions during alcohol rinsing has also been observed with Whatman 3 MM paper and with oil red O dye. Loss of dye during rinsing would indeed be expected from the two phase partition theory of staining, since a new equilibrium of dye distribution between solvent and lipid should be set up with each rinse.

In order to avoid the use of rinsing solutions in which dye is soluble, oil red O stained strips are rinsed briefly in water with agitation. This serves to wash out some of the trapped staining solution before the dye can precipitate on the paper fibers and thus decreases the background by some 20 to 30 per cent without removing an appreciable amount of dye from the lipoprotein bands (Table III).

TABLE III

Effect of water rinsing on the amount of dye recovered from separated alpha and beta lipoprotein fractions stained with oil red O *

Treatment	Dye/2.5 cm. background (*O.D. 520 mμ/5 ml.*)	Net total dye uptake by lipoprotein (*O.D. 520 mμ/5 ml.*)	Alpha lipoprotein dye uptake (*% of total*)
Control	0.261	0.771	23.1
	0.268	0.789	22.2
Rinsed	0.201	0.803	22.2
	0.204	0.791	21.2

* Control strips were blotted immediately after removal from the staining solution.

The chemical significance of lipid staining

Studies of the specificity of lipid staining in the histological literature have led to the conclusion that such dyes as oil red O and the Sudan group are taken up by those phospholipids, neutral fats, and cholesterol esters which are in the liquid state at the temperature of staining and which are not soluble in the dye solvent (18, 21, 22). There is some evidence that different lipids present in a mixture may influence each other's dye uptakes (22). Swahn (9) has shown that the uptake of a preparation of Sudan black by whole serum on filter paper is correlated with the total lipid content of serum; he suggests that the solid lipids such as cholesterol which ordinarily do not take up dye will be stained in serum because of the presence of liquid lipids in which they may be dissolved.

The results of an experiment to test the dye uptake of mixtures of cholesterol and liquid triolein are shown in Table IV. Increasing amounts of cholesterol were added to a solution of triolein in chloroform and the mixtures were applied to paper strips, dried, stained with oil red O, and eluted. Under these conditions cholesterol takes up a negligible quantity of dye and this uptake is not significantly increased by the presence of triolein.

When isolated lipids dissolved in chloroform were applied to paper, dried and stained, it was found that oil red O and Sudan black in 60 per cent ethanol stain triglycerides and lecithin, but that little or no color is obtained with cholesterol, oleic acid, palmitic acid, or ethyl esters of saturated fatty acids with eight to sixteen carbon atoms. Tristearin (m.p. 55°), is stained at 30°, in conflict with the theory that only liquid lipids take up dye.

It is likely that phospholipids in serum also are stained with oil red O since after washing serum strips with acetone at 50°, which is supposed to dissolve all lipids except phospholipids (23), dye is taken up by alpha and beta lipoproteins of normal serum in approximately equal amounts, the total dye uptake amounting to about one-fourth of that in unwashed strips. No appreciable amount of dye is taken up after extraction by hot 2–1 chloroform-methanol.

TABLE IV

Dye uptake by mixtures of triolein and cholesterol

Triolein μg.	Cholesterol μg.	Net dye uptake (O.D. 520 mμ/5 ml.)
125	0	0.243
125	31.3	0.260
125	62.5	0.279
125	125	0.290
0	125	0.023

Reproducibility

In order to obtain any satisfactory reproducibility of staining with lipid dyes it was found necessary to control carefully the temperature during the staining procedure and the composition of the staining solution by close attention to detail in its preparation. The results of a study of the reproducibility of the method as described, under conditions of routine use, are shown in Table V. Analyses were carried out in duplicate on four different normal and abnormal sera which were electrophoretically separated, stained, and eluted on five different occasions over a period of ten days, using a different preparation of dye for each run.

Under these conditions the distribution of dye between the two lipoprotein fractions, as measured by the per cent of the total dye uptake found in the alpha fraction, shows a maximum range of variation of 4.2 per cent (32.6 to 36.8 per cent) in any two individual determinations on the same serum; there is no overlap of the values for the four different sera studied. The total dye uptake shows an almost two-fold range of variation between the highest and lowest values for a single serum in spite of the fact that the concentration of dye in the different staining solutions varied by no more than 6 per cent as shown by measurement of optical density at 520 mμ; there was no relationship between the amount of dye uptake and the concentration of dye in the staining solution. However, if serum P II is arbitrarily selected as a standard with a total dye uptake of 100 units, and the other sera in each run are compared to this standard, the results are more satisfactory with a maximum range of variation of 10 per cent between any two values for the same serum; again there is no overlap of the values for the different sera.

It should be noted that if comparisons are made of the results from duplicate analyses, considerably

TABLE V

Reproducibility of lipoprotein separation and staining of four different sera on five occasions over a 10-day period

Serum	Exp. No.	% Alpha lipoprotein		Net total dye uptake (*O.D. 520 mμ/5 ml.*)		Total dye uptake using P II as standard (arbitrary units)	
P II*	1	22.6	22.7	0.875	0.869	100	99
	2	22.1	21.8	0.742	0.736	100	99
	3	19.2	19.8	0.665	0.660	100	99
	4	20.6	20.3	0.569	0.575	100	101
	5	21.0	21.7	0.708	0.716	100	101
	Average	21.2		0.711		100	
S 1601	1	31.1	31.6	0.999	1.000	114	114
	2	30.5	29.7	0.859	0.878	117	118
	3	28.9	29.6	0.771	0.774	116	116
	4	28.1	27.6	0.648	0.648	114	114
	5	28.5	28.0	0.808	0.839	114	118
	Average	29.4		0.822		115	
S 1605	1	35.9	36.8	0.788	0.779	90	89
	2	35.9	36.5	0.696	0.698	94	94
	3	34.4	35.4	0.608	0.597	91	90
	4	33.8	32.6	0.492	0.482	86	84
	5	32.8	32.9	0.634	0.638	89	90
	Average	34.7		0.641		90	
S 1608	1	12.4	13.7	1.128	1.106	129	126
	2	13.2	12.9	0.991	1.006	134	135
	3	11.5	11.4	0.925	0.907	139	136
	4	11.5	11.3	0.785	0.768	138	135
	5	11.0	10.9	0.976	0.975	138	137
	Average	12.0		0.957		135	

Exp. No.	Concentration of staining solution (*O.D. 520 mμ*)
1	3.840
2	3.936
3	3.804
4	4.068
5	4.008

* Pooled serum from 10 blood donors.

less variation is found. The maximum differences between the results for any two analyses made on the same serum at the same time are 1.3 per cent (12.4 to 13.7 per cent) for the per cent alpha lipoprotein and 0.031 (0.808 to 0.839) optical density units per 5 ml. for the total dye uptake. Since other experiments have shown that strips which are run together but stained separately show a considerable range of variation, it may be inferred that the variations in dye uptake are due to differences in staining with different dye solutions rather than to differences in the electrophoretic separation.

DISCUSSION

The staining of electrophoretically separated lipoproteins by oil red O has the following characteristics; some of these points apply to other staining procedures as well:

1) The increase in dye uptake with increasing lipoprotein concentration is not strictly linear. The resulting variation in the distribution of dye between alpha and beta lipoproteins is, however, no greater than the range of variation of individual determinations on the same serum found in the reproducibility study; thus there will be no appreciable error due to non-linearity in an indi-

vidual determination, but there will be a systematic error in the analysis of large numbers of samples tending towards erroneously low values for beta lipoproteins in high concentration.

2) The solvent for the staining solution, 60 per cent ethanol, dissolves a portion of the lipid from serum lipoproteins in the absence of dye. Whether this occurs in the presence of dye is not known, but it appears likely that there is also a loss of lipid during the ordinary staining procedure. This is not unexpected in view of the known solubility of lipids in alcohol-water solutions under the conditions of histological staining (21). Staining solutions prepared with lower concentrations of alcohol or other solvents have not proved satisfactory in our hands, and indeed there is no reason to believe that other solvents will not dissolve lipid also if they are sufficiently hydrophobic to dissolve the lipid dye. It is therefore necessary to consider that a staining procedure of this type measures only lipid which is insoluble in the dye solvent in the presence of dye.

3) The quantitative chemical significance of the uptake of oil red O by separated lipoproteins is not known. It is possible, by analogy with the staining of whole serum by Sudan black, that dye uptake is correlated with total lipid content of these fractions, but without extensive further investigation of this problem there appears to be no justification for the use of specific chemical terms to express the results of dye binding analysis of separated lipoproteins; the results are best expressed in arbitrary units of dye binding, or as a ratio of dye bound by different fractions.

4) The commercial preparations of oil red O and Sudan black which were examined contained at least four chromatographically distinct colored components. In the case of oil red O at least two components are necessary for satisfactory staining, although the major red component accounts for the majority of the dye actually taken up by lipid. More satisfactory staining with the use of dye mixtures has also been reported with the Sudan dyes in the histological staining of lipids (24). It is quite likely that variations in the proportion of these components in different lots of dye account for the variations in dye uptakes found with different lots. Cain (18) has reported that different preparations of Sudan black exhibit even greater variability in staining ability.

Although the reproducibility of the oil red O method is such that comparisons may be made among sera analyzed with the same lot of dye, it is necessary with different lots of dye to analyze control sera at the same time and under the same conditions to determine normal values. There does not appear to be a valid basis at present for the direct comparison of the results of different laboratories using lipid staining, or for the direct comparison of lipid staining procedures with other methods for measuring lipoproteins.

5) The reproducibility of the distribution of dye between alpha and beta lipoprotein fractions under the conditions described here is probably adequate for most purposes, but the uncorrected total dye uptake shows too much variability on different analyses to be of use as an absolute measure of lipid concentration. However, if a standard serum with a known lipid content determined by other methods is run at the same time as the sera being analyzed, satisfactory values for total dye uptake may be obtained. A known quantity of a stainable lipid could probably also be used as a standard, although the dye uptakes obtained should not be translated directly into terms of lipid concentration because of the uncertainty regarding the specific dye uptakes of different lipids.

6) In our hands the use of 50 per cent ethanol for rinsing of stained separated lipoproteins results in loss of dye from the stained lipoprotein fractions. This is in contrast to the results obtained on rinsing dye from whole serum applied in a spot to dry paper and stained, in which we have confirmed Swahn's observation that little or no dye is lost during rinsing (25). The most obvious difference between the situation with whole serum and separated lipoprotein bands is that the latter are spread over a considerable area of paper and are thoroughly exposed to the rinsing solution, while the former may be protected from loss of dye by incomplete equilibration between dye and rinsing solution. It is also possible that different results may be obtained with different dye preparations or filter papers, and in fact variations in the stoichiometry of dye binding with the use of different papers have been reported (9).

If no rinse is used, or only a water rinse, as described here, most of the background stain on the filter paper cannot be removed. This limits the

selection of dyes that may be used for the staining procedure to those dyes which will give a good stain in a reasonable period of time from a dilute solution; more concentrated dye solutions result in too high a background. Oil red O is a quite satisfactory dye in this respect, giving a comparatively high pattern to background ratio without alcohol rinsing.

It is apparent that the estimation of serum lipoproteins by staining with lipid dyes is not as simple a method as it might at first appear. Before even semi-quantitative validity can be ascribed to the results it is necessary to examine the technique in question with regard to the characteristics noted above. Nevertheless, for the semi-quantitative estimation of these lipoproteins, the staining procedure has the advantages of simplicity, ease of analysis of large numbers of samples, and provision of information as to the mobility and shape of the lipoprotein bands which are not obtained with other methods.

SUMMARY

1. A method is described for the semi-quantitative estimation of serum lipoprotein distribution by means of electrophoresis on filter paper followed by staining of the separated alpha and beta lipoprotein bands with the lipid dye oil red O.

2. This procedure has the following characteristics, some of which apply to other lipid staining procedures as well:

a. Twenty-five to thirty-five per cent of the lipid stained with a solution of dye in 60 per cent ethanol is soluble in 60 per cent ethanol in the absence of dye.

b. There is a deviation from linearity in the relationship between beta lipoprotein concentration and amount of dye bound; this is not of sufficient magnitude to cause a significant error in an individual determination.

c. No dye is lost from lipoprotein on rinsing the stained strips in water; a considerable loss of dye results from rinsing oil red O or Sudan black stained strips in 50 per cent ethanol.

d. The method gives results in terms of dye uptake by lipid insoluble in the staining solution which cannot at this time be directly translated into chemical units.

e. With use of different preparations of a single lot of dye the repeated determination of the lipoprotein distribution of four sera on five occasions shows satisfactory reproducibility; the reproducibility of total dye uptake is not satisfactory without use of a standard with each determination.

f. Commercial dye preparations contain at least four chromatographically distinct colored components; at least two of these are necessary for satisfactory staining. Variations in the composition of different lots of dye result in different staining characteristics of different lots.

3. If used with suitable controls and attention to the details of the procedure, the method described is suitable for the empirical measurement of the distribution and electrophoretic behavior of serum lipoproteins.

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CHOLESTEROL METABOLISM IN MAN

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It has been previously demonstrated that, in the rat, the major metabolic pathway of cholesterol involves the conversion of this molecule to bile acids (1, 2). These are excreted into bile and after repeated enterohepatic circulation (3) are finally eliminated in the feces, still in the form of bile acids. Over 80 per cent of the body's cholesterol was found to be metabolized in this manner (1, 2).

In rat bile, the chief bile acid resulting from cholesterol metabolism has been identified specifically as the taurine conjugate of cholic acid (4–6).

Since it is known that this reaction is not limited to the rat (7), there is some reason to expect that cholesterol is metabolized primarily by way of bile acids in man as well. Quantitative studies of the pathways of cholesterol breakdown in human beings have not, however, been previously published. Hellman and his co-workers have administered C^{14}-labeled cholesterol to patients and stated that this sterol is converted to cholic acid and to steroid hormones; however, no information on the quantitative importance of these compounds in cholesterol metabolism was reported (8).

The purpose of the present study was to assess the quantitative significance of the various routes of cholesterol excretion and to determine the major end-products of cholesterol metabolism in man.

MATERIAL AND METHODS

Cholesterol-4-C^{14}

The labeled cholesterol was obtained from Radioactive Products, Detroit, Mich., and had a specific activity of 5 μC per mg. Before use the cholesterol was purified by digitonin precipitation and chromatography on alumina as previously described (6). The sterol was administered intravenously as a Tween 20, saline solution (9).

Treatment of patient

The subject of this investigation was a 54-year-old male who three weeks prior to beginning the study was subjected to a cholecystectomy (diagnosis: cholecystitis and cholelithiasis). At the time of surgery a T-tube was inserted into his common bile duct. An inflatable balloon located below the distal arm of the T allowed complete drainage of bile to the exterior when desired.

The experiment was initiated by inflating the balloon and immediately injecting intravenously 3 microcuries (600 γ) of cholesterol-4-C^{14}. All bile was then collected for a period of 50 hours. At the end of this time the balloon was deflated and bile was again allowed to enter the duodenum. Feces were saved during the 50 hours of biliary drainage and for 6 days thereafter. Urine was likewise obtained both during the period of bile collection and for the following 40 hours. All samples were frozen immediately after collection and were stored at $-10°$ C. until analyzed. A single, half-hour sample of expired air was collected 29 hours after the cholesterol administration.

Bile

Total biliary C^{14} was assayed by directly mounting an aliquot of bile on an aluminum planchet and counting the dried sample in a Robinson-type flow-gas counter. All counts were corrected for self absorption.

Total bile acid-C^{14}

Bile acid-C^{14} was determined as described previously (2). Samples of the bile were saponified in 4 N NaOH at 125° C. for 2 hours. They were shaken three times with approximately a tenfold excess of petroleum ether to extract the radioactivity contained as nonsaponifiable compounds. Following acidification the bile was again shaken with petroleum ether to extract fatty acids. Then ethyl ether extraction was used to obtain the bile acid fraction which was finally purified through the Doubilet procedure (10) to yield total bile acid-C^{14}.

Isolation of bile acids by use of the carrier technique

In order to determine the quantitative importance of a specific bile acid involved in cholesterol excretion, carrier amounts (100 to 500 mg.) of the pure nonradioactive bile acid were added to the bile sample. This was then saponified at 125° C. in 4 N NaOH for 2 hours. The solution containing saponified C^{14}-bile acids and the carrier bile acid was next acidified and the resulting gummy precipitate removed, washed with water, and dried *in vacuo*. Cholic acid was recrystallized directly from this gum by using ethyl acetate as the solvent. Desoxycholic acid was isolated by crystallizing from methyl ethyl ketone, and recrystallizing from acetone.

Weighed portions of the crystals were counted after being redissolved in ethanol and dried in the planchets.

Chromatography

Paper chromatography of the bile was carried out with Whatman No. 1 filter paper in a collidine, ammonia, water system as previously described (6). The bile acids were visualized by spraying the dried chromatogram with 50 per cent antimony trichloride in glacial acetic acid. When an estimate of the C^{14} content of the bile acid spots was desired, one-quarter inch strips on either side of the chromatogram were sprayed with antimony trichloride, to determine the positions of the bile acids on the paper. The bile acids in the unsprayed, central portion of the chromatogram were then eluted directly into planchets and the eluate was dried and counted.

Feces

Feces were extracted with three portions of boiling ethyl alcohol for a total period of one hour. The residue was then placed in a Soxhlet apparatus and extracted for 12 more hours with ethyl alcohol. An aliquot of the pooled extracts was counted without further purification.

Urine

Aliquots of urine were plated directly and counted in the Robinson counter.

RESULTS

Routes of excretion of cholesterol end-products

During the course of the 50-hour collection period 39.0 per cent of the administered C^{14} was recovered in the subject's bile (Figure 1). It was felt inadvisable to deprive the patient of his bile for a longer period of time; however, it seems reasonable to assume from the curve shown in Figure 1 that cholesterol end-products would have continued to be eliminated by this route at a comparable rate.

That bile does constitute the chief route of cholesterol excretion in man is strongly suggested by comparing the results of C^{14} analysis of the bile with that of the acholic feces, urine and expired air. In the same time period during which 39 per cent of the injected C^{14} was being eliminated in the bile, only 0.5 per cent of the cholesterol-4-C^{14} was found in the feces and 0.1 per cent in the urine. No $C^{14}O_2$ was detected in the expired air. As summarized in Table I, therefore, it is clear that over 98 per cent of the excreted C^{14} was eliminated by way of bile.

The feces and urine samples obtained after the 50-hour period of bile collection were found to contain, respectively, 4.1 and 0.06 per cent of the administered cholesterol-C^{14}.

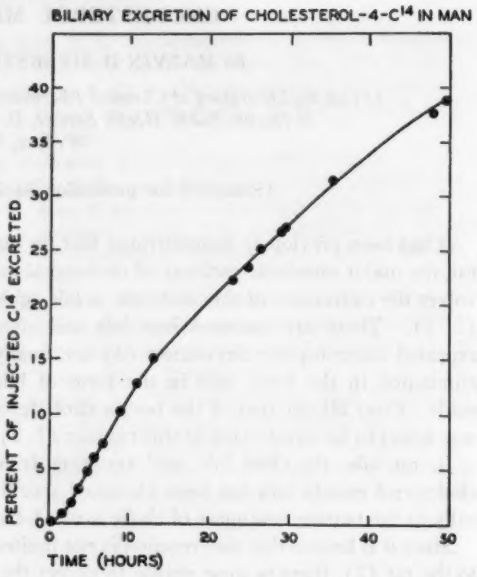

FIG. 1. CUMULATIVE EXCRETION OF C^{14} INTO BILE FOLLOWING INJECTION OF CHOLESTEROL-4-C^{14}

Nature of the biliary end-products of cholesterol metabolism

The C^{14}-containing end-products of cholesterol metabolism were identified by the three methods described in the experimental section.

1. *Isolation of (a) the nonsaponifiable fraction which includes cholesterol, dihydrocholesterol and coprosterol; (b) the fatty acid fraction; and (c) the total bile acid fraction.* Assay of these three fractions indicated (Table II) that cholesterol is excreted in bile primarily as a bile acid(s). In

TABLE I

Routes of excretion of cholesterol-4-C^{14} in man *

Source	Per cent of excreted C^{14}
Bile	98.5
Acholic feces	1.3
Urine	0.2
CO_2	0.0

* Period of collection was 50 hours, except for the CO_2 collection where a single one-half hour sample was taken. Total C^{14} excretion was 39.6 per cent of the administered C^{14}.

TABLE II

Nature of the biliary end-products of cholesterol metabolism

Time period (*hrs.*)	Fraction	Per cent of total biliary C^{14} in fraction
3–4	Nonsaponifiable	11.2
	Fatty acid	.1
	Bile acid	88.7
7–9	Nonsaponifiable	7.6
	Fatty acid	.2
	Bile acid	92.2
29–36	Nonsaponifiable	10.2
	Fatty acid	.4
	Bile acid	89.4
36–48	Nonsaponifiable	7.0
	Fatty acid	.4
	Bile acid	92.6

the four bile acid samples analyzed only about 10 per cent of the C^{14} was found in the nonsaponifiable fraction; negligible activity was present in the form of fatty acids. On the other hand, between 89 and 93 per cent of the biliary C^{14} was found in the bile acids, isolated by ethyl ether extraction and precipitation according to the Doubilet procedure.

2. *Isolation by the carrier technique.* Identification of the specific bile acids to which cholesterol is converted was accomplished by adding known bile acids to the bile, followed by recrystallization of the recovered bile acids to constant specific activity (see Experimental). It was found (Table III) that most of the radioactivity was present in bile as cholic acid. Desoxycholic acid was found to comprise a small per cent of the cholesterol excretion products in both of the samples analyzed.

3. *Paper chromatography.* Paper chromatography of a bile sample collected between the 36th and 48th hours was performed in order to confirm the results of the above two procedures and also to further identify the major cholesterol end-products in bile.

TABLE III

Identification of the bile acid end-products of cholesterol metabolism as isolated by the carrier technique

Time period (*hrs.*)	Per cent of total C^{14} of sample in cholic acid + desoxycholic acid	Per cent of total bile acid C^{14} in	
		CA	DCA
29–36	87	90	7
36–48	90	90	7

On spraying a chromatogram of human bile with the antimony trichloride solution four bands became visible (Figure 2). The first with an R_f of 0.49 to 0.53 was by far the darkest and was identified with pure standards as glycocholic acid. Immediately above the glycocholic acid was seen a band giving the purple color of a dihydroxylated bile acid. It is likely that this is the glycine conjugate of the desoxycholic acid isolated by the carrier technique since taurodesoxycholic migrates much faster than the observed band. This band no doubt also contains glycochenodesoxycholic acid. At an R_f of 0.75 a small amount of taurocholic acid color is usually visible. Finally, cholesterol and perhaps monohydroxy bile acids are seen at the front.

The C^{14} content of these bands could be assayed only approximately since there is undoubtedly some overlapping of activity on the paper. The results are given in Figure 2. The major portion of the C^{14} was definitely present in the glycocholic acid band with the small amounts of C^{14} in the taurocholic acid, glycodesoxycholic acid and at the front making up the remainder of the activity.

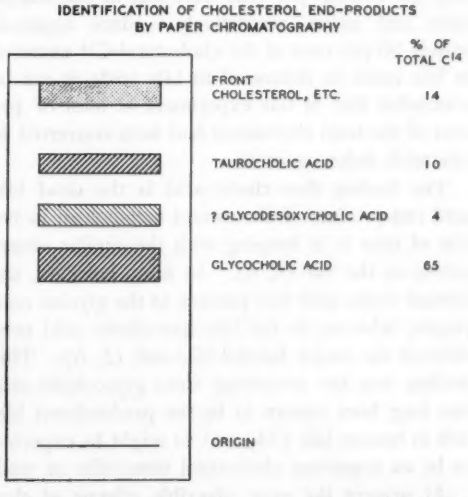

FIG. 2. SCHEMATIC DRAWING OF A PAPER CHROMATOGRAM OF BILE SAMPLE TAKEN FROM THE 36th TO THE 48th HOUR AFTER CHOLESTEROL-4-C^{14} INJECTION

For details see text.

These results are in good agreement with those obtained by use of the carrier technique.

DISCUSSION

Until recently it has been the general opinion that the 27-carbon neutral sterol, coprosterol, was the major end-product of cholesterol metabolism. Cholesterol itself and dihydrocholesterol were believed to make up most of the remainder of the body's sterol excretion while bile acid synthesis was assumed to play a relatively minor role in this process (11–13).

That coprosterol is not the chief end-product of cholesterol metabolism has now been amply shown in the rat. Further, it has been demonstrated in this species that most of the body cholesterol is metabolized by way of bile acids and that it is primarily as bile acids that cholesterol is excreted into both the bile and the feces (1, 2).

Evidence that man, too, converts a large fraction of his body cholesterol to bile acids before excretion is presented here.

In a human subject given cholesterol-4-C^{14}, 39.6 per cent of the administered sterol was found to be eliminated by all routes of excretion in a 50-hour period. Of this amount, however, fully 98 per cent was excreted by way of bile while only 1.3 and 0.2 per cent were lost in the acholic feces and urine, respectively. Since approximately 90 per cent of the cholesterol-C^{14} excreted in bile could be recovered as bile acids, it can be concluded that in this experiment at least 87 per cent of the total cholesterol had been converted to bile acids before excretion.

The finding that cholic acid is the chief bile acid end-product of cholesterol metabolism in the bile of man is in keeping with the similar observation in the rat (4, 6). In man, however, the labeled cholic acid was present as the glycine conjugate, whereas in rat bile taurocholic acid constituted the major labeled bile salt (5, 6). This finding was not surprising since glycocholic acid has long been known to be the predominant bile salt in human bile (14) and so might be expected to be an important cholesterol metabolite as well.

At present the most plausible scheme of cholesterol breakdown in the rat involves the conversion of cholesterol to desoxycholic acid (15) and the subsequent hydroxylation of desoxycholic acid to yield cholic acid (16). The demonstration in the present study that cholesterol can be converted to desoxycholic acid as well as to cholic acid is consistent with the idea that such a pathway may also be present in man.

Finally the inability of the human body to metabolize cholesterol-4-C^{14} to $C^{14}O_2$ also agrees with previous observations that neither the rat nor the dog can perform this oxidation (1, 9, 17) and confirms the similar finding by Hellman and his co-workers (8, 18) in man.

SUMMARY

1. Cholesterol-4-C^{14} was injected intravenously into a man with complete biliary drainage. The routes of excretion and the major compounds to which cholesterol is converted were then studied.

2. Of the administered cholesterol 39.6 per cent was eliminated by all routes of excretion in a 50-hour period.

3. Over 98 per cent of the excreted cholesterol appeared in the bile, 1.3 per cent in the acholic feces, and 0.2 per cent in the urine. No $C^{14}O_2$ was found in the expired air.

4. It has been shown that in man the major pathway of cholesterol metabolism involves the conversion of cholesterol to bile acids.

5. The chief excretory product of cholesterol in the bile of man has been identified as glycocholic acid.

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CHANGES IN THE PHYSICAL CHARACTERISTICS OF THE HYALURONATE OF GROUND SUBSTANCE WITH ALTERATIONS IN SODIUM CHLORIDE CONCENTRATION [1]

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Hyaluronic acid was first isolated in 1934 by Meyer and Palmer (1) from the vitreous humor of the cattle eye. It has since been isolated from the ground substance of some connective tissues, synovial fluid, cartilage, cornea, umbilical cord, bacteria, and possibly identified in the ground substance of other material (2, 3). The substance is a high polymer with a monomer of a disaccharide, β-glucuronido-1-3 N-acetylglucosamine, constituting the repeating unit (4). Each of these monomers contains an ionizable carboxyl group. The compound is one of a large group of natural polyelectrolytes.

The connective tissue, in the ground substance of which hyaluronic acid is found, is a ubiquitous material, and thought to be of importance in support, ion transport, storage, and repair (3). Changes in the physical characteristics of hyaluronic acid may be of importance in the exercise of these functions.

Studies of the physical properties of hyaluronic acid by various methods have been reported (5–14). We have studied the relative changes in certain biophysical measurements of hyaluronate solutions including flow birefringence, viscosity, sedimentation and light scattering as a function of sodium chloride concentration to determine the role of the sodium ion in such physical changes.

Methods and results. Studies of flow birefringence, viscosity, sedimentation and light scattering were made on solutions of sodium hyaluronate at various concentrations of NaCl maintained at pH 5.0 with acetate buffer. This value was selected in order that the studies be performed at a pH optimal for the action of hyaluronidase, in anticipation of further enzymatic studies. From electrometric titrations, the material is estimated to be about 80 per cent ionized at this pH (1, 15). Hyaluronate from two sources was studied: human umbilical cord and streptococcal capsule. They were prepared by an alcohol precipitation method (16). Proteolytic digestion was used in the umbilical cord specimen. Chemical analyses of the two materials are shown in Table I, and show that the preparations are essentially protein-free.

A complete series of measurements were made using the umbilical cord specimen and confirmatory determinations for the streptococcal hyaluronate were also performed. The relative changes in the measurements were the same in both specimens and, for the sake of brevity, the values for umbilical hyaluronate only will be shown.

Flow birefringence. Flow birefringence on solutions at 0.25 per cent concentration of hyaluro-

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TABLE I

Chemical analysis of hyaluronate specimens

	Nitrogen	Hexosamine	Sulfate	Hexos./ nitrogen	Uronic acid (Dische)	Uronic acid/ nitrogen
Human umbilical cord MV 62	3.03%	38.0%	<0.4	0.98	45.5%	1.08
Strep. capsule MV 69A	3.06%	40.9%	—	1.05	44.0%	1.04

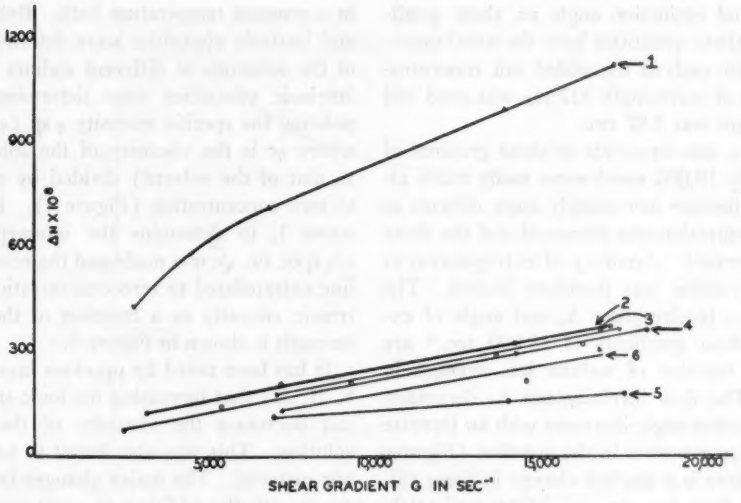

FIG. 1. FLOW BIREFRINGENCE, Δ_n

Human umbilical cord hyaluronate concentration 0.25 per cent.

Flow birefringence. Δ_n as a function of shear gradient for solutions at various sodium chloride concentrations at pH = 5.0 (acetate buffer).

Sodium ion strength; curve 1, 0.005; curve 2, 0.085; curve 3, 0.115; curve 4, 0.25; curve 5, 1.6; curve 6, 2.25.

nate was determined at room temperature on the small flow birefringence instrument described by Edsall, Rich, and Goldstein (17). Measurements of angular velocity were made with a Strobotac lamp. Shear gradients up to 19,000 sec.$^{-1}$ were obtained. Extrapolations to zero shear gradient and to 18,000 sec.$^{-1}$ were made for purposes of comparing the different solutions. Figures 1 and

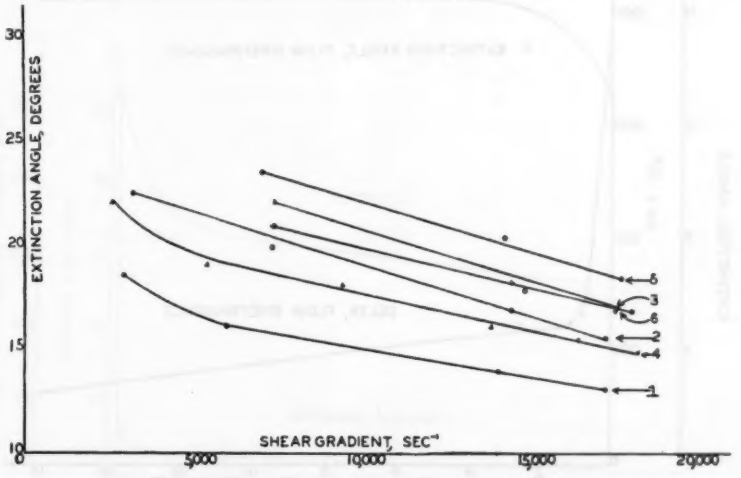

FIG. 2. FLOW BIREFRINGENCE, EXTINCTION ANGLE

Human umbilical cord hyaluronate concentration 0.25 per cent.

Flow birefringence extinction angle as a function of shear gradient for solutions at various sodium chloride concentrations at pH 5.0 (acetate buffer).

Numbering of curves as in Figure 1.

2 show Δ_n and extinction angle *vs.* shear gradients (where these quantities have the usual meanings [18]) for each of the added salt concentrations. Light of wavelength 517 mμ was used and the path length was 3.87 cm.

In all cases, measurements at shear gradient of approximately 18,000 sec.$^{-1}$ were easily made although they became increasingly more difficult as the salt concentration was increased and the shear gradient decreased. Accuracy of extrapolation to zero shear gradient was therefore limited. The value for flow birefringence Δ_n and angle of extinction at shear gradients of 18,000 sec.$^{-1}$ are shown as a function of sodium ion strength in Figure 3. The flow birefringence Δ_n decreases, and the extinction angle increases with an increase in the salt concentration in the solution (Figures 1, 2, 3). There is a marked change in these values below sodium ion strength of approximately 1.3 and only small changes with addition of salt beyond this concentration.

Viscosity. Viscosity was measured in an Ubbelöhde (19) viscometer with a capacity of 3.75 cc. and a distilled water flow time of 126 seconds. The apparatus was maintained at 37° C. ± 0.05 in a constant temperature bath. Relative, specific and intrinsic viscosities were determined for each of the solutions of different sodium ion strength. Intrinsic viscosities were determined by extrapolating the specific viscosity η sp (η sp = η r − 1, where ηr is the viscosity of the solution relative to that of the solvent) divided by concentration, to zero concentration (Figure 4). In the case of curve 1, to determine the intercept, a plot of c/η spec. *vs.* $\sqrt{c}$ was made and the resultant straight line extrapolated to zero concentration (20). Intrinsic viscosity as a function of the sodium ion strength is shown in Figure 5.

It has been noted by previous investigators (5–8, 21, 22) that increasing the ionic strength of the salt decreases the viscosity of the hyaluronate solution. This was also found to be the case for our material. The major changes in the viscosity occur with the addition of small amounts of salt. After approximately 0.5 M, the drop is gradual and after 1.3 M, there is very little change with the addition of even large amounts of salt.

Balazs and Laurent (8) have shown by measurements of intrinsic viscosity as a function of concentration of hyaluronate that the material be-

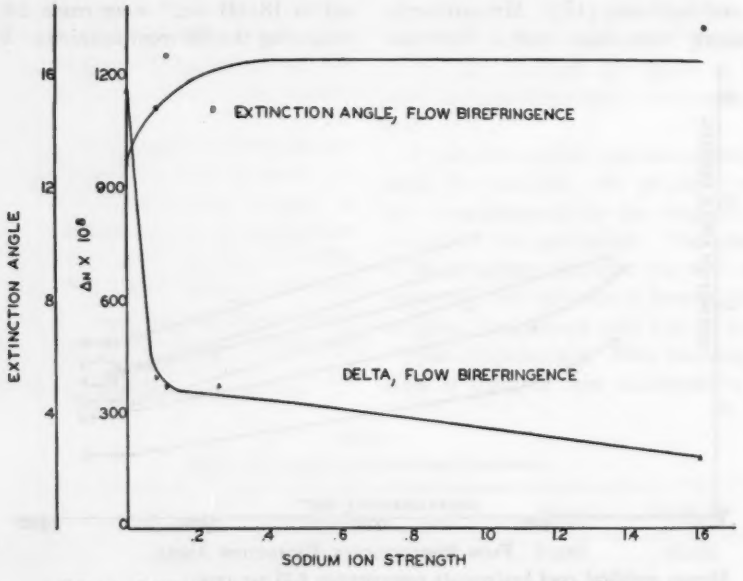

FIG. 3. FLOW BIREFRINGENCE AS A FUNCTION OF SODIUM ION STRENGTH

Human unbilical cord hyaluronate concentration 0.25 per cent. pH = 5.0 (acetate buffer).

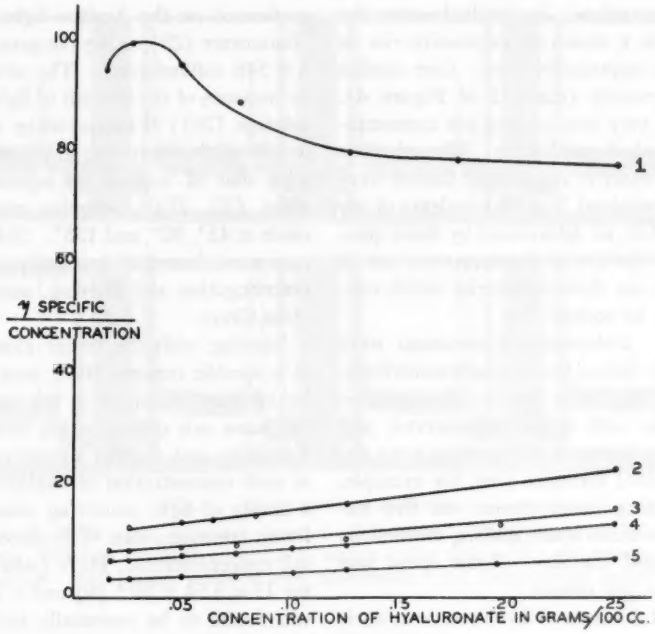

FIG. 4. VISCOSITY STUDIES

Human umbilical cord hyaluronate.

η spec./conc. as a function of concentration of hyaluronate, for solutions at various sodium chloride concentrations at pH = 5.0 (acetate buffer).

Sodium ion strength; curve 1, .005; curve 2, .025; curve 3, .085; curve 4, .115; curve 5, 1.6.

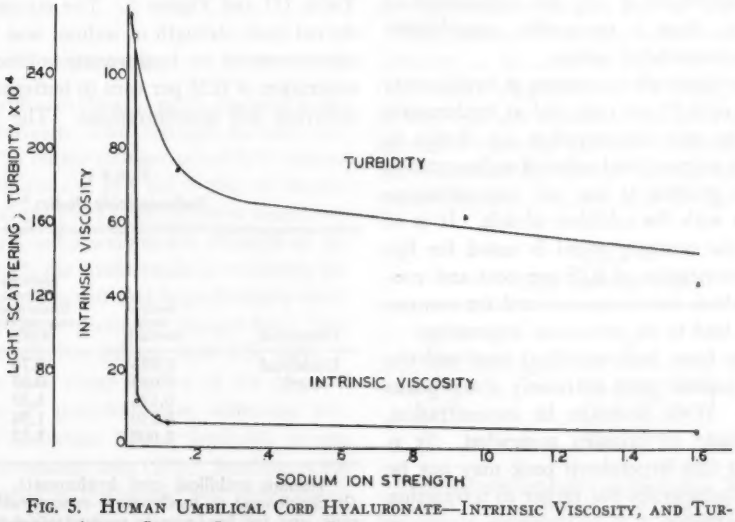

FIG. 5. HUMAN UMBILICAL CORD HYALURONATE—INTRINSIC VISCOSITY, AND TURBIDITY (FROM LIGHT SCATTERING STUDIES) AS A FUNCTION OF SODIUM ION STRENGTH

haves as a polyelectrolyte. In distilled water, the curve of η sp/c *vs.* c shows an impressive rise as the concentration approaches zero. Our studies confirmed their results (curve 1 of Figure 4). They achieved a very low sodium ion concentration by dialysis and electrodialysis. The solutions made in distilled water in the present studies were undialyzed and contained 5 milliequivalents of sodium ion per 100 cc. as determined by flame photometry. Hence, the rise in the curve was not as striking as it was for Balazs' material which contained essentially no sodium ion.

Sedimentation. Sedimentation constants were determined on the Spinco model E ultracentrifuge. Measurements of the peaks on the photographic plates were made with a microcomparator and by projection. Sedimentation constants were determined by standard formulae (see, for example, reference 23), using measurements on five exposures, each 32 minutes apart, making corrections for temperature and viscosity. Rotor speed was 59,780 revolutions per minute.

Runs at several dilutions of hyaluronate at various salt concentrations were performed and sedimentation at infinite dilution determined by extrapolating 1/S *vs.* c by the method of Ogston (24), where S is the sedimentation constant in Svedbergs and C is the concentration of hyaluronate in grams per 100 cc. Since the sedimentation is difficult to determine at very low concentrations of hyaluronate, there is necessarily considerable error in the extrapolated values.

Values of sedimentation constant at hyaluronate concentration of 0.25 per cent and at hyaluronate extrapolated to zero concentration are shown in Table II. The extrapolated value of sedimentation appears to be greatest at low salt concentrations and decreases with the addition of salt. It is of interest that the contrary trend is noted for hyaluronate concentration of 0.25 per cent and consideration of these values uncorrected for concentration might lead to an erroneous impression.

Hyaluronate from both umbilical cord and the streptococcal capsule gave extremely sharp peaks (Figure 6). With decrease in concentration, these were found to broaden somewhat. It is suggested that this hypersharp peak may not be due to low polydispersity but rather to interaction between the particles.

Light scattering. Light scattering studies were performed on the Aminco light scattering microphotometer (25), using the green line of mercury, $\lambda = 546$ millimicrons. The absolute turbidity τ (a measure of the amount of light scattered by the solution [26]) is measured by comparing the intensity of the scattering at an angle of 90 degrees with that of Ludox, an aqueous suspension of silica (25, 27). Angular measurements were made at 45°, 90°, and 135°. Solutions of hyaluronate were cleared of dust and particulate matter by centrifugation and filtering, using suitable fritted glass filters.

Starting with the buffer alone containing salt at a specific concentration, measured amounts of hyaluronate dissolved in the same buffer and at the same salt concentration were added serially. Turbidity and angular measurements were made at each concentration of hyaluronate. This gives a family of light scattering measurements at different concentrations of hyaluronate for different salt concentrations. Hc/τ (where the optical factor $H = 3.54 \times 10^{-6}$ [9] and c is in gm. per cc.) was found to be essentially independent of concentration for each of these solutions in a range from 3×10^{-4} to 9×10^{-4} gm. per cc. of hyaluronate. From these curves a value for turbidity corrected for dissymmetry at hyaluronate concentration of 5×10^{-4} gm. per cc. was determined and is shown as a function of sodium ion strength in Table III and Figure 5. The corrected turbidity *versus* ionic strength of sodium was rechecked by measurements on hyaluronate solutions at a concentration of 0.25 per cent in buffered solutions of different salt concentrations. The turbidity de-

TABLE II

Sedimentation studies *

Preparation	Sodium ionic strength	Sedimentation at hyaluronate concentration 0.25%	Sedimentation for concentration of hyaluronate extrapolated to zero
Umbilical cord	0.025	0.76	6.3
	0.085	0.89	4.2
	0.175	1.39	
	0.250	1.54	2.8
	1.600	1.52	

* Human umbilical cord hyaluronate. Sedimentation (in Svedbergs) at hyaluronate concentration of 0.25 per cent, and for hyaluronate concentration extrapolated to zero, in solutions containing sodium chloride at various concentrations (pH 5.0 acetate buffer).

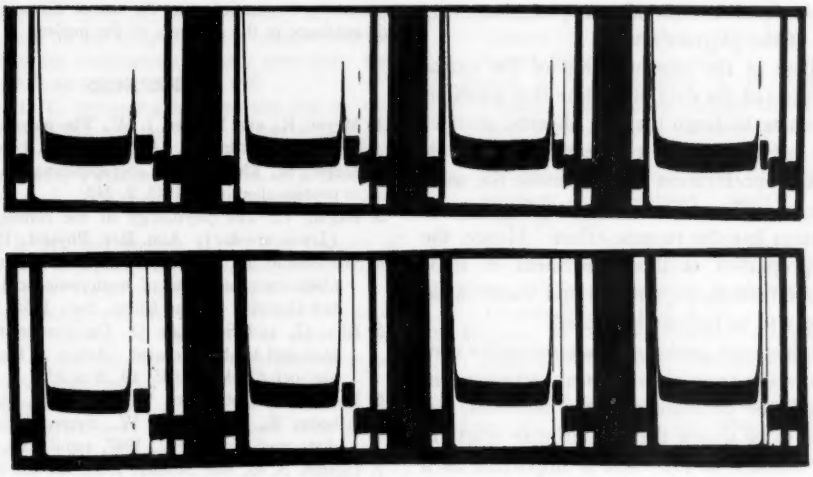

FIG. 6. ULTRACENTRIFUGE STUDIES

Rotor speed 59,780 rpm. Exposure interval, 32 minutes. Concentration of hyaluronate 0.5 per cent. Solutions are made up in 0.1 M acetate buffer (pH = 5.0) and contain 0.15 M sodium chloride. (a) Human umbilical cord hyaluronate. (b) Streptococcal capsule hyaluronate.

creases with increase in sodium chloride concentration. The major decrease occurs below a sodium ion strength of 1.5 and changes only slightly with changes in salt concentration beyond this value. The dissymmetry measured at 45° decreases slightly with the salt concentration. See Table III.

DISCUSSION

There are profound changes in the physical properties of hyaluronate with changes in salt concentration. The major changes occur in a sodium ion concentration range just below that of physiological saline where there is an exquisite sensitivity to this ion. Beyond a sodium ion strength of approximately 0.15, the hyaluronate is relatively insensitive. It is of interest that in preliminary studies, similar but more profound changes have been found with chondroitin sulfuric acid (28, 29).

There have been many studies of the physical characteristics of polyelectrolyte solutions, *i.e.*, high polymer molecules having ionizable groups as part of the repeating unit (30). Studies on the chemical structure of hyaluronic acid have shown it to fit into this classification (4). The present data on its behavior in salt solutions are, in general, what one would expect of such a charged polymer.

It should be understood that interpretation of such data in terms of physical models can be only approximate and general. Some remarks, however, can be made about the probable changes in the hyaluronate particle and its solutions.

The increase in sedimentation constant and in turbidity as the ionic strength is decreased below 0.15 molar suggests that the particles aggregate in this low salt concentration environment. The increased viscosity and flow birefringence at low salt concentration further strengthens this hypothesis, although consideration of these values alone

TABLE III

Light scattering studies *

Sodium ion strength	τ_{90} corrected	Dissymmetry at 45°
0.005	28×10^{-3}	4.84
0.145	19×10^{-3}	4.43
0.915	17×10^{-3}	4.35
1.6	13×10^{-3}	4.38

* Human umbilical cord hyaluronate. Turbidity at 90° corrected for dissymmetry, and dissymmetry at 45°, for hyaluronate concentration of 5×10^{-4} gram per cc. in solutions containing sodium chloride at various concentrations (pH 5.0 acetate buffer).

would be compatible with other explanations, *e.g.*, uncoiling of the polymer chain.

Regardless of the interpretation of the extrapolated values of the data, it is clear that solutions of hyaluronate undergo marked physical changes when the salt concentration is changed. Decreasing the salt concentration tends to make the solution more "rigid" and "stiff." Increasing the salt concentration has the reverse effect. Hence, the physical properties of this constituent of some of the ground substance, and salt and water variations appear to be intimately related.

Our studies were performed *in vitro* under conditions differing from physiological as to concentration, pH, the presence of other materials, etc. It remains to be shown that hyaluronate reacts in a similar manner *in vivo* and is important as a factor in the control of salt and water in the body as in edema, inflammation, dehydration and other states in which water may be shifted between body compartments.

SUMMARY

1. Biophysical studies on two specimens of isolated hyaluronate in solutions containing different concentrations of sodium chloride have been performed. The studies include flow birefringence, viscosity, sedimentation and light scattering.

2. There are marked changes in these measurements with change in sodium ion strength. The values (extrapolated to infinite dilution of hyaluronate) suggest that the particles aggregate at low salt concentrations and disaggregate with the addition of salt. These changes occur in a region of physiological interest.

3. There is an intimate relationship between the physical properties of hyaluronate solutions and sodium chloride concentration. If this material acts in a similar manner *in vivo*, it may be of importance as a factor in the control of salt and water metabolism as in edema, inflammation, dehydration and other states in which water may be shifted between body compartments.

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INCREASED ELECTROPHORETIC MOBILITY OF ALBUMIN IN THE SERUM OF RATS SUBJECTED TO CARBON-TETRACHLORIDE TREATMENT OR TO TOURNIQUET SHOCK

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It has been previously reported (1) that binding of small quantities of certain fatty acids to serum albumin causes a decrease of its azorubin-binding capacity (ABC). It was assumed then that the low ABC of serum albumin observed in clinical cases (2) or in experimental animals (3, 4) was produced by a firm binding, *in vivo*, of increased amounts of certain anions, possibly higher fatty acids.

If this explanation were correct, a negative increment in the net charge of the albumin molecule would be expected. The electrophoretic mobilities of albumin were therefore determined in sera of rats subjected to carbon-tetrachloride poisoning and to tourniquet shock since these conditions are known to lower the ABC of serum albumin (3). Studies on the influence of dialysis on the ABC of sera and other protein solutions were included as supporting data.

EXPERIMENTAL

Animal experiments. Male Sprague-Dawley rats were given i.p. injections of 0.5 ml. CCl_4 per Kg. body weight every second day for six days and twice this volume of a 1 + 1 mixture of CCl_4 and ethanol on the eighth day. The blood was obtained on the tenth day. Other details have been given previously (3). The average weight of the rats declined from 336 gm. to 271 gm. under the injections of CCl_4. Tourniquet shock was produced by applying rubber bands to both hind legs (3). The average weight of the animals at the time of sacrificing was 269 gm. The blood of all experimental animals was drawn from the heart and the serum prepared as described (3). The average non-protein nitrogen concentration in the sera listed in Table I was found to be 72 mg. per cent, indicating a severe state of shock. The various samples of serum for the normal, CCl_4-treated and tourniquet-shock rats were obtained from a total of 88, 163, and 148 animals, respectively.

Chemical procedures. Total protein and non-protein nitrogen were determined by a micro-Kjeldahl procedure as outlined previously (3).

Electrophoretic analysis. The sera were dialyzed against sodium phosphate buffer of pH 7.7, $\mu = 0.1$, for a total of 22 hr. at 2° C. in a mechanically stirred dialyzer (5) with six changes of buffer. The diluted serum solution was used for the measurement of the pH (glass electrode, room temperature) and resistivity. The electrophoretic analyses were done in a standard size electrophoresis apparatus [1] using the long analytical cell. The separations were mostly run for 120 minutes at + 0.4° C. and 24 mA. The conductivity was measured in a Shedlovsky cell equilibrated at + 0.4° C.

The evaluation of the electrophoretic patterns was done as in earlier studies (3), using the descending boundaries only. The procedure of Longsworth and MacInnes (6) was applied to determine the distance traveled by albumin. As a check of the accuracy of the electrophoretic analysis, the mobility of crystalline bovine serum albumin (Armour) was determined in a 1 per cent solution in glycine sodium hydroxide buffer, $\mu = 0.2$ at pH 8.4. The value obtained (-5.79×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8.35) was in agreement with the mobility (-5.8×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8.4) reported in the literature (7).

Dialysis experiments. All dialyses were performed at 2° C. ± 0.5° C. The solutions of fatty acids in human albumin and the control samples were prepared as previously described (1). They were dialyzed in a mechanically stirred dialyzer (5) with buffer changes and for the time periods given in Table III. At pH 7.7, phosphate (0.02 μ)-sodium chloride (0.08 μ) buffer was used; at the higher pH values, glycine-sodium hydroxide-sodium chloride buffer of ionic strength 0.2 (8) was employed. The dialysis periods at pH 11.5 and 12.0 (Table III) were followed by a dialysis of about 16 hrs. against the phosphate-sodium chloride buffer at pH 7.7 with several changes of buffer. The final control and experimental solutions were adjusted to the same volume. This procedure assured an equal final ionic composition and approximately the same total dialysis times for all experiments of Table III. The determination of the ABC by a chromatographic procedure on anionotropic aluminum oxide and the calculation of the number of moles of fatty acid bound per mole albumin have been outlined previously (1).

For the dialysis of sera at neutral pH, distilled water, 0.6 per cent sodium chloride and the above phosphate-sodium chloride buffer were used. The dialyses at higher pH values were done against 0.1 μ glycine buffer (8), followed by phosphate-sodium chloride buffer, pH 7.7,

[1] Frank Pearson Associates, New York 12, N. Y.

TABLE I

Electrophoretic mobilities of albumin in rat sera

Average Values

Condition of rats	No. of determinations	Total protein in gm. %	−u Observed in 10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}	−u Corrected for 1.58% protein in electr. cell	−u Corrected for 1.76% protein in electr. cell
Normal	11	6.64	5.78	5.76	5.67
S.E. ±				0.032	0.033
F.L. ±				0.07*	0.06†
CCl_4 Treated	10	6.04	5.95	5.97	—
S.E. ±				0.057	
F.L. ±				0.13*	
Tourniquet Shock	5	5.90	5.78	—	5.88
S.E. ±					0.074
F.L. ±					0.16†

* Fiducial limits for probability level 0.95.
† Fiducial limits for probability level 0.90.

to complete equilibration. The dialysis times ranged between one and 3 days at pH values up to 9.5; at pH 11.5, the sera were dialyzed for 5½ hrs.

The various dialysates of the sera were acidified and extracted several times with petroleum ether. The dry residues of the petroleum-ether phase were combined with human serum albumin as described before (1). For the direct extractions of normal and low-ABC sera, alcohol-ether mixtures (3:1) were used at normal and elevated temperatures; the lipid material was also combined with albumin.

RESULTS AND DISCUSSION

The results of the electrophoretic analysis are given in Table I.[2] All essential requirements for accurate determinations of mobility, as outlined by Alberty (10), have been met in the present study. A correction was applied to the mobility values in order to account for the somewhat lower serum protein concentration observed in the injured rats (3, and Table I). The mobility value of a protein is influenced by the concentration of all proteins present, mainly through changes in viscosity. However, since mere correction for viscosity results in an overcorrection (10), the adjustment was made on the basis of experimental findings correlating the electrophoretic mobility with protein concentration. An interpolation of the data that Lippman and Banovitz (11) obtained for albumin in rat serum gave a dependency of the mobility (u) on the protein concentration (c), expressed in gm. per cent, $\Delta u/\Delta c = -0.46 \times 10^{-5}$. The same quotient was found in the present studies for albumin in rat serum (phosphate buffer, pH 7.7, $\mu = 0.1$) and was adopted for the calculations.

[2] Unabridged tables and a more detailed discussion have been given elsewhere (9).

TABLE II

Influence of pH on electrophoretic mobility of albumin in normal rat serum

Total protein concentration in serum: 6.33%
Total protein concentration in electrophoretic cell: 2.11%
Phosphate buffer, $\mu = 0.1$. Descending boundaries.

pH	−u in 10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}	$-\Delta u/\Delta pH \times 10^5$	$-\Delta u/\Delta pH \times 10^{5*}$
6.10	4.39		
7.68	5.58	0.75	0.75
8.48	6.00	0.53	0.50

* Values obtained by graphic interpolation of data given by Miller, Miller, and Eitelman (Figure 4 in [12]) for albumin in human plasma.

TABLE III

Influence of dialysis of albumin-fatty acid mixtures on ABC

Fatty acid	Dialysis: pH	Dialysis: Hours	Dialysis: Changes of buffer	Decrease of ABC %	Corresponding to moles of fatty acid per mole albumin
	Before dialysis			44	2.3
Lauric	7.7	22	6	44	2.3
	11.5	5	5	32	1.5
	12.0	7	7	20	0.9
	Before dialysis			53	23
Caproic	7.7	22	6	10	1
	11.5	5	5	8	1

The corrections were made to the medium protein concentrations of the groups of sera to be compared with each other. In this way the extent of corrections was reduced to a minimum (for further details see [9]).

Since no information seemed to be available for the influence of pH on electrophoretic mobility of albumin in rat serum, these data were determined at three pH values. Table II shows that the quotients $\Delta u/\Delta pH$ obtained were almost identical with the values calculated by interpolation from results of Miller, Miller, and Eitelman (12) for albumin in human plasma. An application of the data of Table II to the mobility values of Table I showed that the hydrogen ion concentrations were sufficiently close to make corrections unnecessary (for the magnitude of such corrections see [9]).

In order to test the significance of differences between mobility values found in different sera, the reproducibility of the individual observations was checked. Duplicate determinations were made of the anodic mobilities of albumin in four (diluted) sera from normal rats and from rats treated with CCl_4. The results of the eight duplicate analyses showed that the deviations of the two duplicate observations from their medium values ranged from 0 to 0.8 per cent, the average deviation being 0.44 per cent, S.E. $= \pm 0.078$ per cent (F.L. 0.01 $= \pm 0.23$ per cent). This means that the reliability of the mobility values observed (Table I) is such that at a 0.99 probability level the statistical deviation from the "true" values is not greater than 0.025×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

The data of Table I indicate that the albumin in the serum of CCl_4-treated rats had a greater anodic electrophoretic mobility than the albumin in normal serum. The difference averaged 0.21×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} and was found to be statistically significant at a probability level of 0.95. The albumin mobility in the serum of rats subjected to tourniquet shock was also found to be greater than normal. The anodic mobility of the albumin in the sera of acidotic and x-irradiated rats (13) did not differ from the normal values (for details see [9]).

These results demonstrate that statistically significant elevations of the anodic mobility of albumin were found in all those sera that showed significant decreases in the ABC (sera from rats treated with carbon-tetrachloride or subjected to tourniquet shock), whereas normal albumin mobilities were observed in the sera with normal ABC (sera from acidotic and x-irradiated rats).

The difference between the anodic mobilities of albumin in the sera of normal and CCl_4-treated rats was found to be about 0.2×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} (Table I). Since no data seemed to be available for the relation between electrophoretic mobility and net charge for rat serum albumin, the findings of Longsworth and Jacobsen (14) on the influence of net charge (z) on the mobility (u) of bovine serum albumin were utilized. The relationship was found to be a linear one, the slope of the curve being $\Delta u/\Delta z = 0.2 \times 10^{-5}$ (Figure 4 in [14]). On the assumption that the ratio $\Delta u/\Delta z$ for rat serum albumin is similar to that for bovine albumin it is concluded that the average albumin molecule in the serum of CCl_4-treated rats differs from that of normal rats by carrying about one more negative charge.

The possession of approximately one more negative charge on the albumin molecule of low-ABC sera is in agreement with the results obtained previously (1) on the combination of albumin with anions. After *in vitro* addition of the order of one to two moles of higher fatty acids per mole human serum albumin or albumin in rat serum, the ABC decreased by approximately the same degree as observed in CCl_4-treated and tourniquet-shock rats (3). On the basis of the extent of binding of various fatty acids by serum albumin (15) it is probable that the combination of the small amounts of fatty acids added to albumin to produce this decrease of the ABC (1) is essentially complete. The observation (Table I) of a higher negative net charge in the albumin of sera with lower ABC, therefore, corroborates the previous suggestion that a decrease in the ABC values is caused by a firm binding *in vivo* of increased amounts of certain anions, *e.g.*, fatty acids, to the albumin molecules.[3] The numerical value

[3] It should be mentioned that the serum from rats treated with CCl_4 was found to contain more non-esterified higher fatty acids firmly bound to albumin (1.30 moles, S.E. $= \pm 0.06$, per mole albumin, calculated as monocarboxylic acid) than that from normal rats (0.85 mole, S.E. $= \pm 0.04$). These analytical studies will be published elsewhere (see also G. L. Selden and U. F. Westphal, Concentration of non-esterified higher fatty acids in serum of carbon tetrachloride-treated and normal rats, and in rabbit and bovine serum. Army Medical Re-

of the difference in the net charge is in agreement with the assumption that one additional anion is firmly attached to the albumin molecule in the low-ABC sera. No greater accuracy, however, should be ascribed to this value than that of an order of magnitude.

The possibility was investigated that the amount of anions, *e.g.*, higher fatty acids, bound to albumin in the original serum would be altered by the dialysis. Table III shows that the decrease of ABC observed after dialysis of a human albumin-lauric acid mixture at pH 7.7 was the same as before, indicating that no change in the amount of bound lauric acid (1) took place. It is concluded that the amounts of anions firmly bound to albumin, *e.g.*, higher fatty acids, in the dialyzed sera used in the electrophoretic analysis (Table I) are the same as in the original sera. In accordance with the general experience that the firmness of binding between albumin and fatty acids decreases when their chain lengths become shorter (16), a substantial amount of caproic acid was found to be removed from albumin under the same conditions of dialysis (Table III).

The conclusions drawn from the dialysis experiments on albumin-lauric acid mixtures at pH 7.7, were confirmed by studies on sera. Normal sera and sera of low ABC were dialyzed at neutral pH in an attempt to reduce or eliminate the difference between the ABC values of the two types of sera. The difference remained unchanged. Equally unsuccessful was the endeavor to isolate material from normal and low-ABC sera at neutral pH by dialysis or extraction with organic solvents, to combine the material from the two types of sera with pure human albumin and thus demonstrate different influences on the ABC. In accordance with the dialysis experiments (Table III), these conditions appear to be too mild to affect the firm combinations of albumin and anions that are believed to influence the ABC of albumin in sera.

Dialysis of sera of normal and "tourniquet-shock rats" at pH 9.5 reduced the difference between the ABC values from an average of 27 per cent (3) to 7 to 8 per cent. Dialysis at pH 11.5 completely abolished the difference in the ABC of albumin between normal and "tourniquet-shock" sera. A corresponding result was obtained with the mixture of albumin and lauric acid: dialysis at pH 11.5 and 12.0 reduced the decrease of the ABC value from 44 per cent to 32 and 20 per cent, respectively (Table III). The explanation for these results is believed to be a removal of firmly bound anions from albumin by dialysis at the higher pH values. This interpretation is in accordance with the studies of Klotz, Walker, and Urquhart (17, 18) on the influence of pH on the binding of azosulfathiazole and methyl orange to bovine serum albumin.

It was observed in the present studies that the gradual disappearance of the differences between the ABC of normal and low-ABC solutions by dialysis at increased pH was accompanied by a general decline of the absolute ABC values to about three-fourths or two-thirds of the original. This general reduction is considered to be indicative of a partial denaturation of the albumin (19). However, under the conditions employed, the ABC was not completely destroyed; *e.g.*, dialysis at pH 12 for 5 hrs. reduced the ABC value of a normal human albumin solution to 73 per cent of the original. Subsequent addition of 2.3 moles of lauric acid per mole albumin caused a further decrease of the reduced ABC value by 47 per cent which is close to the 44 per cent observed in untreated albumin (Table III). In the light of these model studies it is concluded that the disappearance of the differences between the ABC of albumin in normal and low-ABC sera results from the removal of firmly bound anions, *e.g.*, higher fatty acids, by dialysis at the higher pH.

The results obtained on the electrophoretic mobilities of albumin in the sera of normal and injured rats have a direct bearing on the specific problem of the ABC. Besides, however, they appear to be of interest in view of the great number of data that were obtained in many laboratories on the electrophoretic mobility of albumin in normal and pathological human sera. A systematic analysis of such albumin mobility values, determined under strictly identical conditions, may assist in detecting abnormalities in the amounts of anions, *e.g.*, higher fatty acids, firmly bound to

search Laboratory Report No. 182, 10 March 1955 and also see G. L. Selden and U. Westphal, Non-esterified higher fatty acids in serum of CCl_4-treated and normal rats and other species. Proc. Soc. Exper. Biol. & Med., 1955, **89**, 159).

albumin and thus contribute to an elucidation of biochemical changes that occur in human blood under certain pathological conditions.

SUMMARY

1. The electrophoretic mobility of albumin in the serum of rats subjected to carbon-tetrachloride poisoning or tourniquet shock was found to be higher than that of the albumin in normal rat serum. The difference corresponded to an increment of approximately one negative charge per albumin molecule.

2. Elevated anodic mobilities were observed only for serum albumin of decreased azorubin-binding capacity (ABC), indicating the firm binding of an increased amount of anions to the albumin in sera of low ABC. The numerical value of the difference in net charge corresponded to a quantity of additionally bound anions that would decrease the ABC, according to model studies, to a similar degree as found for low-ABC albumin.

3. The difference between the ABC values of normal and low-ABC albumin or serum was reduced or eliminated by dialysis at pH 11.5.

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THE TRANSPORT OF LIPIDS IN CHYLE[1]

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Recent evidence favors the concept that with the exception of short chain fatty acids nearly all dietary fat is delivered from the intestinal tract to serum by way of the thoracic duct (1, 2). Patients with thoracic duct fistulae provide an opportunity to examine lipids in a state of transition between the native form in which they are ingested, and the state in which they exist in the clear serum of fasting normal individuals. Chyle is of particular interest because it is a physiological fluid rich in neutral fat (3) which in turn has a profound influence on the physical state of lipids in serum (4).

The purpose of the present study was to determine the concentration and physical state of the lipids in chyle. The lipids were divided into two categories: those present in the emulsified state causing the milky appearance of chyle, and those present in clear solution. This separation was easily effected by a relatively brief and slow-speed ultracentrifugation, which concentrated the emulsified lipids causing lactescence in a creamy layer at the top of the centrifuge tube, leaving the remainder of the lipids present in the clear subnatant fluid (4).

METHODS

Chyle was collected from patients with chylothoraces secondary to mediastinal carcinoma or to pulmonary surgery, or with external thoracic duct fistulae in the neck secondary to surgery for carcinoma. In addition, several lactescent-appearing ascitic fluids were examined. No patients had fasted longer than overnight prior to collection of the chyle. No effort was made to estimate the completeness with which chyle was excluded from the blood stream, though it is doubtful if it was ever complete. Unless otherwise stated the chyle collected had accumulated over an unknown period of time. Serum lipids were determined in each patient at least once. Blood for this determination was drawn when the patients were in the post-absorptive state.

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Chyle was obtained from two dogs by cannulating the thoracic duct. Both dogs were anesthetized with Nembutal®. The thoracic duct was approached through the bed of the eighth rib on the right, and a polyethylene catheter was inserted just above the ampulla. The free end of the catheter was brought to the surface and the wound was closed. Between collections of chyle the catheter was filled with physiological saline to maintain patency, and clamped off at the distal end. The use of heparin was strictly avoided.

All specimens of chyle were treated in the same manner. After removal of any cells or protein clots by slow-speed centrifugation, the undiluted chyle was centrifuged at 18,000 rpm, for one hour in a Spinco Model L preparative ultracentrifuge. This was performed at room temperature to avoid possible breakdown of lipoproteins in the cold. There was no appreciable rise in the temperature of the material in the centrifuge. This procedure removed by flotation the lipids present in the emulsified state, leaving the clear subnatant fluid beneath the cream layer. The subnatant fluid so obtained was carefully removed, using a technique previously described (4).

Lipid analyses were carried out on the original chyle and on the clear subnatant fluid. The concentration of the emulsified lipids, which was estimated as the difference between the lipids in original chyle and those in the subnatant fluid, is given in terms of their concentration in the original chyle rather than in a top layer of varying or unknown volume. It was assumed for this estimation that the lipids in the clear subnatant fluid were nearly uniformly distributed throughout the centrifuge tube. The justification for this assumption has been discussed (4). The lipids measured were total fatty acids, total cholesterol, lipid phosphorus, and in some instances free cholesterol. The methods used have been described elsewhere (5). Fatty acids are expressed in milliequivalents per liter, cholesterol in milligrams per cent and phospholipids as milligrams per cent of lipid phosphorus, the terms in which they are measured. Neutral fat was calculated indirectly from the above data (5) and is expressed as milliequivalents per liter of neutral fat fatty acids. For this calculation it is assumed that fatty acids not in cholesterol esters or phospholipids are in neutral fat, and that no fatty acids exist in the free form. When free cholesterol was not determined it was assumed for this calculation that 72 per cent of the cholesterol was esterified, the average figure for normal serum (5).

Total protein was determined, using the biuret method for clear fluid, and the Kjeldahl method for cloudy

fluid. In one instance the emulsified lipids of chyle were washed free of water soluble substances by replacing the subnatant fluid with saline, recentrifuging, and repeating the procedure until three washings had been completed. The concentrations of lipids and protein in the washed lipid layer were determined.

Free fatty acids were determined in a few instances by evaporating the alcohol-ether extract to near-dryness on a warm electric plate well guarded by wire gauze. A stream of nitrogen was bubbled through the extract during the latter part of the evaporation to avoid oxidation of fatty acid chains or destruction of phospholipids. The residue was immediately taken up in alcohol, brought to a boil, and titrated directly with 0.02 N sodium hydroxide under a stream of carbon dioxide-free air.

RESULTS

Thoracic duct chyle or chylous pleural fluid was obtained in 13 instances from five patients having either external thoracic duct fistulae or chylothoraces. All specimens were milky in appearance. The lipid and protein values of chyle and of serum obtained at approximately the same time are shown in Table I. The neutral fat of chyle varied from 24 to 294 mEq. per L., the cholesterol from 14 to 97 mg. per cent, and the lipid phosphorus from 4.4 to 23 mg. per cent. Twenty-one to 38 per cent of the cholesterol was present in the free form. The protein was between 2.72 and 4.49 gm. per cent. The higher concentration of protein in the subnatant than in the original chyle may be partly explained by dilution of the original chyle with protein-poor particulate lipid. The lipids of the corresponding sera were not remarkable except for a tendency to be low.

The lipids of chyle were present largely in the emulsified state, indicated in Table I as the difference between the subnatant fluid and the original chyle. Neutral fat constituted the bulk of the particulate matter, but it was also found in the clear subnatant fluid in concentrations which, though relatively small, were quite constant, varying from 6.0 to 10.8 mEq. per L. despite great variations of the total neutral fat. Cholesterol and phospholipids were divided between the particulate and the soluble fractions. From 50 to 87 per cent of the cholesterol and from 41 to 77 per cent of the phospholipids were present in the emulsified form. The ratios of free to total cholesterol were similar in both groups of lipids and in serum. The greater variability of the ratios in chyle may be due in part to the technical difficulty in determining cholesterol when it is present in such low concentrations.

In one patient (No. 4) it was possible to note the effect of a specific meal on chyle collected from a freely flowing thoracic duct fistula in the neck. The first specimen of April 6, 1954 was collected from 7:30 AM to 8:30 AM. Although this patient had fasted for 12 hours, the specimen was milky in appearance and contained 35 mEq. per L. of neutral fat. The next specimen was collected from 8:30 to 11:30 AM. At the beginning of this period the patient ate a light breakfast consisting of one egg, a glass of skimmed milk, a banana and a slice of dry toast. The emulsified neutral fat increased to 62 mEq. per L. and the lipid phosphorus also rose sharply, but the cholesterol did not change significantly.

In this same patient (No. 4) the effect of diet on chyle was observed. The first three specimens were obtained while the patient was receiving a high protein diet containing 70 gm. of fat a day. At noon on April 6 the patient was placed on a diet containing 30 gm. of fat a day. Chyle collected between 24 and 48 hours after the institution of this diet contained only 24 mEq. per L. of neutral fat, even less than the fasting specimen of April 6. While the chyle became less lactescent in appearance it never became clear.

Free fatty acids determined in the subnatant fluid of patient No. 4 on April 5 and April 8 were 0.0 and 0.6 mEq. per L. The free fatty acids of the original chyle of patient No. 4 on April 5 were 1.1 mEq. per L., and of patient No. 5 were 1.7 mEq. per L. Since the presence of water-soluble organic acids was not ruled out these figures are maximal and indicate that free fatty acids do not exist in significant quantities in chyle.

The results of analysis of ascitic fluid which appeared somewhat lactescent are shown in Table II. Despite the cloudy appearance, the fluid otherwise bore little resemblance to chyle, having very little lipid or protein, the cloudiness evidently resulting chiefly from a small amount of neutral fat, most of which was removed by ultracentrifugation. Even though the serum fatty acids of one patient (No. 7), a nephrotic, were elevated to 94.9 mEq. per L., the fatty acids of the ascitic fluid were only 2.9 mEq. per L.

The lipid and protein values of thoracic duct

TABLE I

Lipids of chyle and serum before and after ultracentrifugation

Patient	Unit number / Age / Sex	Date		Chyle: Total fatty acids	Chyle: Cholesterol Total	Chyle: Cholesterol Free	Chyle: Cholesterol Free/Total	Chyle: Lipid phosphorus	Chyle: Neutral fat fatty acids	Chyle: Protein	Serum: Total fatty acids	Serum: Cholesterol Total	Serum: Cholesterol Free	Serum: Cholesterol Free/Total	Serum: Lipid phosphorus	Serum: Neutral fat fatty acids	Serum: Protein	Comment
				mEq./L.	*mg. %*	*mg. %*	*%*	*mg. %*	*mEq./L.*	*gm. %*	*mEq./L.*	*mg. %*	*mg. %*	*%*	*mg. %*	*mEq./L.*	*gm. %*	
1	36-72-06 68 M	11/14/52	Original	40.2	92	33	36	6.5	35.0	2.98	8.4	133	43	32	6.4	2.4	5.35	Chylothorax secondary to mediastinal lymphoma
			Subnatant	9.8	21	8	38	2.5	7.9	3.40								
			Difference	30.4	71	25	35	4.0	27.1									
		11/17/52	Original	49.9	76	17	22	6.2	44.8	2.72								
			Subnatant	10.4	22	8	36	2.8	8.4	3.07								
			Difference	39.5	54	9	17	3.4	36.4									
2	39-12-62 58 M	8/3/53	Original	79.1	78	16.5	21	11.8	70.7	3.91	8.9	116	24	21	7.0	2.6	6.53 on 7/30	Chylothorax secondary to pneumonectomy for chronic lung abscess
			Subnatant	12.7	12			4.7	9.8	4.14								
			Difference	66.4	66			7.1	60.9									
		8/7/53	Original	50.9	72			7.5	45.2	4.49	11.6	150			8.6	4.0		
			Subnatant	10.7	16			3.9	8.1		10.8	127	44	35	8.2	3.6		
			Difference	40.2	56			3.6	37.1		0.8	23			0.4	0.4		
		8/9/53	Original	47.0	59			7.4	41.7									
			Subnatant	9.9	11			3.2	7.9									
			Difference	37.1	48			4.2	33.8									
		8/29/53	Original	308.1	14.5			23.0	294.5								6.64	
			Subnatant	11.8	14.0			5.3	8.4									
			Difference	296.3	0.5			17.7	286.1									
		9/30/53	Original	102.0	64			12.5	93.7								7.82 on 9/28	
			Subnatant	10.0	9			3.5	7.8									
			Difference	92.0	55			9.0	85.9									
3	A68337 63 F	8/13/53	Original	112.0	97			14.9	101.7		10.8	166			7.4	3.4		Chyle obtained from thoracic duct fistula in neck 2 hours after lunch. Epidermoid carcinoma with metastasis in neck
			Subnatant	10.4	49			5.4	6.4	4.36								
			Difference	101.6	48			9.5	95.3									
4	40-43-01 50 M	4/5/54 6:00 AM to 1:00 PM	Original	87.7	76	27	36	10.9	80.2		10.6	151			7.0	3.7	5.54 alb. 2.43 glob. 3.11	Thoracic duct fistula in neck secondary to radical neck dissection for metastatic carcinoma. On 70 gm. fat, high protein diet
			Subnatant	10.1	25			3.9	7.4	3.27								
			Difference	77.6	51			7.0	72.8	alb. 1.79 glob. 1.48								
		4/6/54 7:30 to 8:30 AM	Original	41.1	88			7.6	35.3									Fasting
			Subnatant	8.8	40			3.8	6.0									
			Difference	32.3	48			3.8	29.3									
		4/6/54 8:30 to 11:30 AM	Original	67.7	79			10.0	62.2									Light breakfast at 8:30 AM
			Subnatant	8.8	30			3.9	6.0									
			Difference	58.9	49			6.1	56.2									
		4/7/54 4/8/54	Original	27.7	49			4.4	24.3									At noon, 4/6, started on low fat diet (30 gm. per day)
			Subnatant	12.5	18			2.4	10.8									
			Difference	15.2	31			2.0	13.5									
5	41-24-65 60 F	9/13/54	Original	31.9	97	26	27	5.4	27.0		12.3	174			7.9	4.5		Pleural effusion; multiple thromboembolism of unknown cause
			Subnatant	8.5	28	8	28	3.2	6.2	4.32								
			Difference	23.4	69	18	26	2.2	20.8									

TABLE II

Lipids of ascitic fluid and serum before and after ultracentrifugation

Patient	Unit number / Age / Sex	Date		Total fatty acids	Cholesterol: Total	Cholesterol: Free	Cholesterol: Free/Total	Lipid phosphorus	Neutral fat fatty acids	Protein	Material	Comment
				mEq./L.	*mg. %*	*mg. %*	*%*	*mg. %*	*mEq./L.*	*gm. %*		
6	B29384 48 M	1/28/53	Original	11.8	214	106	50	10.0	3.2	5.25	Serum	Cirrhosis with ascites
		1/29/53	Original	3.4	16.0	7.3	46	1.4	2.3	0.87	Ascitic fluid	
			Subnatant	0.9	6.8			1.1	0.7			
			Difference	2.5	9.2			0.3	1.6			
7	40-99-40 34 F	6/23/54	Original	94.9	838	263	31	22.3	64.8	3.32	Serum	Nephrosis with hyperlipemia
			Subnatant	32.7	322	114	35	14.2	19.1			
			Difference	62.2	516	149	29	8.1	45.7			
		6/23/54	Original	2.9	11.2	3.5	31	1.0	2.1	0.29	Ascitic fluid	
			Subnatant	0.8	2.9			0.9	0.3			
			Difference	2.1	8.3			0.1	1.8			
8	C37082 27 F	11/17/54	Original	4.4	16	4.5	28	1.4	3.3	0.74	Ascitic fluid	Nephrosis with clear serum
			Subnatant	1.5	7							
			Difference	2.9								
		11/18/54	Original	4.6	75.6					3.55	Serum	
9	B49958 62 F	1/26/54	Original	9.0	141	56	40	6.8	2.1	5.08	Serum	Cirrhosis with ascites
		1/26/54	Original	4.4	17			1.7	3.1		Ascitic fluid	
			Subnatant	1.6	8			1.5	0.5			
			Difference	2.8	9			0.2	2.6			

chyle obtained from two dogs are given in Table III, together with other pertinent data. The first dog had fasted for 12 hours and had previously received a diet of Purina laboratory chow containing 5.6 per cent fat. The second dog had ingested nothing but water and glucose for 72 hours. All specimens were only slightly opalescent. The initial specimen of each dog contained 9.6 and 8.7 mEq. per L. of neutral fat, much less than did the specimens from human patients whose dietary fat was much greater.

Of particular interest was the increase in opalescence and in neutral fat of the cream in chyle obtained from dog No. 2, 22 hours postoperatively

TABLE III

Lipids of chyle from fasting dogs before and after ultracentrifugation

Dog	Date		Total fatty acids	Cholesterol: Total	Cholesterol: Free	Cholesterol: Free/Total	Lipid phosphorus	Neutral fat fatty acids	Protein	Rate of flow	Comment
			mEq./L.	*mg. %*	*mg. %*	*%*	*mg. %*	*mEq./L.*	*gm. %*	*ml./hour*	
1	4/20/53	Original	16.2	97			8.2	9.6	3.95	28	14.9 Kg. dog, 12-hour fast Chyle collected during operation
		Subnatant	10.4	90			7.4	4.4			
		Difference	5.8	7			0.8	5.2			
2	5/6/53 12:40 to 1:40 PM	Original	13.5	69	20	29	6.2	8.7	2.79	25	8.9 Kg. dog, ingested only glucose and water for previous 72 hours
		Subnatant	5.4	41				<5.4			
		Difference	8.1	28							
	3:40 to 4:10 PM	Original	3.1	17.5				<3.1	1.46	52	500 ml. saline infusion just completed
		Subnatant	2.0	16.0				<2.0			
		Difference	1.1	1.5							
	5/7/53 10:15 to 11:45 AM	Original	6.6	14.7	4.6	31	1.5	5.5	0.78	42	Immediately following 450 ml. saline and glucose by stomach tube
		Subnatant	2.5	13.7	3.4	25	1.2	1.5			
		Difference	4.1	1.0	1.2		0.3	4.0			

over that obtained three hours after the cannulation. The animal had been offered only glucose and water in the interim and for three days previously. This increment followed the administration of 450 ml. of 0.85 per cent saline and 5 per cent glucose by stomach tube. The protein and cholesterol dropped progressively in the chyle of this dog, becoming too low to measure with accuracy.

In one patient the emulsified lipids of the chyle, when washed free of the subnatant fluid, contained 81.9 mEq. per L. of total fatty acids, 118 mg. per cent of cholesterol, 9.9 mg. per cent of lipid phosphorus, and 74 mEq. per L. of neutral fat fatty acids. Despite the high concentration of lipids, the concentration of protein was only 0.15 gm. per cent, well within the error of the method, 0.3 gm. per cent.

DISCUSSION

Thoracic duct lymph provides an important link in the transport of massive quantities of lipids in the emulsified state, originating chiefly in the diet, to sites of lipoprotein formation remote from the intestinal tract. These lipids are the product of the large-scale hydrolysis and resynthesis to which dietary fat is subjected during digestion and absorption. When alimentation is normal they constitute the bulk of lipids present in chyle, and cause its lactescent appearance.

Neutral fat is the predominant element of chyle in the non-fasting state. The rise and fall of neutral fat in response to fat-feeding and fat-restriction were shown in the chyle of patient No. 4. The chyle of two dogs after the complete withdrawal of fat from their diets was only slightly opalescent, and the neutral fat was much lower than in the chyle of normally eating human patients. Also in contrast to the human patients, the cholesterol and phospholipids occurred chiefly in the clear subnatant layer (Table III). Chyle from a previously reported infant with chylothorax became clear after a week of parenteral non-fat feeding, and the neutral fat dropped to 3 mEq. per L. (3).

The interrelations between neutral fat, cholesterol, and phospholipid are shown in Figure 1, where the concentration of each lipid element in both the soluble and emulsified form is plotted against total neutral fat. In the chyle of the human patients the concentration of phospholipid was directly related to that of neutral fat, a relationship occurring chiefly in the emulsified lipids. This association is consistent with the known concept of phospholipid as a product of digestion. It indicates that a rather constant proportion of ingested fat in an unrestricted diet is converted to phospholipid during absorption and transported with the emulsified fraction of chyle.

No relationship was noted between cholesterol and either the neutral fat or phospholipid of chyle. There did seem to be an inverse relationship between the cholesterol in the clear subnatant fluid and that in the emulsified form (Figure 1). It has been suggested that the former is endogenous and the latter largely dietary (6). The above relationship may thus reflect the reciprocity between the rate of synthesis of endogenous cho-

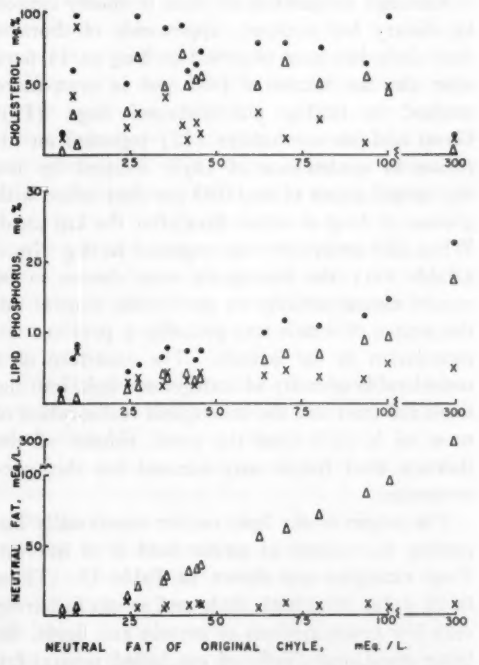

FIG. 1. RELATIONSHIP OF THE VARIOUS LIPID COMPONENTS OF CHYLE TO THE TOTAL NEUTRAL FAT

The fraction of each occurring in the emulsified and in the soluble state as well as the total concentration of cholesterol and lipid phosphorus are shown. Total ●. Soluble X. Emulsified Δ.

lesterol by the liver and the intake of dietary cholesterol (7).

The ratios of free to total cholesterol in chyle, when they were determined, were similar to those of serum. This was true of the cholesterol in both the emulsified form and in clear solution (Table I). The ability of the intestinal tract to esterify cholesterol has been demonstrated (2, 8, 9) and Swell and Treadwell (10) have defined the specificity of pancreatic cholesterol esterase.

The cholesterol and phospholipid found in the particulate layer of chyle are probably dissolved in the neutral fat particles, the emulsification of which may be aided by the phospholipid. Their subsequent "clearing" may thus depend on the dissolution of the neutral fat. Evidence has been presented suggesting that the cholesterol and phospholipids present in the "cream" layer of lactescent sera are dissolved in the neutral fat particles which fail to be "cleared" in these states (4).

Although lactescence of chyle is chiefly caused by dietary fat, a cloudy appearance of thoracic duct chyle has been observed as long as 14 days after the last intake of food and is even more marked in fasting phlorhizinized dogs (11). Glenn and his co-workers (12) reported an increase in opalescence of chyle induced by the chylogogic action of oral 0.85 per cent saline with glucose as long as seven days after the last meal. When this procedure was repeated in dog No. 2 (Table III) the lactescence was shown to be caused almost entirely by particulate neutral fat, the source of which was probably a previous accumulation in the lacteals. The excretion of a considerable quantity of endogenous lipid into the intestinal tract and the subsequent reabsorption of most of it (13) into the small volume of the thoracic duct lymph may account for this phenomenon.

The origin of the lipid matter occasionally imparting lactescence to ascitic fluid is of interest. Four examples are shown in Table II. These fluids differ from both chyle and serum in having very low concentrations of protein and lipids, the latter consisting chiefly of emulsified neutral fat. Glenn and his co-workers (12) noted a slight escape of dye from lymphatics to blood by routes other than the thoracic duct. This escape was immediately increased by obstruction of the thoracic duct. A similar escape of lipids from the intestinal lymphatics to the interstitial spaces, possibly exaggerated by pressure of the ascites on the thoracic duct, might account for the lactescence of some ascitic fluid. Such fluid is ordinarily limpid, and contains low concentrations of proteins and lipids (14).

The lipids in the cream layer of chyle, like those of lactescent serum (4, 15) contain very little, if any, protein. For this reason it seems likely that the large lipid particles responsible for lactescence are present in their native state of insolubility in water, not in any association with protein other than that which may adhere to the surface of such emulsified particles. The lipids present in the clear subnatant layer, on the other hand, have been rendered water soluble by their combination with certain proteins to form lipoproteins. While these may be caused to rise at different rates after prolonged ultracentrifugation in media of artificially increased density (16, 17), they remain undisturbed in the clear subnatant fluid in the present study.

The lactescent appearance of chyle is usually so striking that the characteristics of the soluble lipoproteins are obscured, but after centrifugation they may be studied in the clear subnatant fluid. The source of these lipoproteins of chyle is not known. They could conceivably arise from any region drained by the thoracic duct. Page, Lewis, and Plahl (18) reported the presence in thoracic duct lymph of dogs of lipoproteins similar to those in plasma, but in lower concentrations, suggesting the serum as a source of some of the lipoproteins. The liver lymph must also be considered an important, possibly a major source of these lipoproteins. The concentrations of protein (19) and cholesterol (8) are greater in liver lymph than in peripheral lymph. Forker, Chaikoff, and Reinhardt (20) demonstrated in rats that a large fraction of protein synthesized by the liver is delivered to the thoracic duct lymph, and in dogs that as much as half of the serum proteins circulate through the thoracic duct daily. Bollman showed that intravenously injected dextran appears in the thoracic duct within minutes, chiefly by way of the liver lymphatics (21), and Waddell, Geyer, Clarke, and Stare demonstrated that similarly injected fat emulsions are removed chiefly by the liver (22). To the liver is attributed the major role of lipoprotein formation.

It seems fair to surmise that while the emulsified lipids of chyle originate in the intestinal lymphatics, the lipoproteins in the subnatant fluid are derived chiefly from liver lymph.[2] When dietary fat is withheld the interrelations of chyle lipids are similar to those of serum (3), and may represent the nature of lipoproteins released from the liver under these circumstances.

On the other hand, the subnatant lipids of chyle in the fat fed state differ markedly from those of serum (Table I). The concentration of cholesterol is very much lower, and that of neutral fat higher than are their respective concentrations in serum. This discrepancy could be explained if it were assumed that the lipids of the subnatant fluid of chyle reflect the nature of serum lipoproteins as they are first formed, but that the lipoproteins in serum have been subjected also to the influence of different rates of peripheral utilization or removal of the individual lipids. The fact that neutral fat is relatively a much larger component of the lipoproteins as they occur in the thoracic duct than as they occur in serum may indicate that the peripheral tissues remove neutral fat with great rapidity, and cholesterol and phospholipid more slowly.

This concept is in agreement with newer concepts of lipid metabolism. In the past, the belief was widely held that since most tissues are not permeable to neutral fat or cholesterol esters, the only function of neutral fat was the storage of fatty acids, and that only phospholipids could serve to transport fatty acids destined to be burned. More recently it has been shown by isotope studies that in contrast to the slow turnover of phospholipid and cholesterol in serum, the turnover of neutral fat fatty acids is extremely rapid in humans (23) as well as in animals (24). Hellman, Rosenfeld, and Gallagher (25), in long term isotope studies in humans, noted that after an initial rapid decay, the half-life of cholesterol in serum might be as long as 32 days. While serum cholesterol has other functions not directly related to fat metabolism, such as the formation of steroid hormones and bile acids (26), its role in fat metabolism, if it has any, appears to be mainly one of facilitating the transport and utilization of neutral fat, a role in which it is not itself used up with any rapidity.

These findings and those of the present study suggest that a cholesterol-phospholipid-protein matrix acts as a carrier system for conveying neutral fat in the soluble form from the liver to the peripheral tissues. This shuttle system would continue until all the insoluble lipid particles temporarily deposited in the liver after a fat meal were removed. The rate at which the liver disposed of these particles would depend, according to this hypothesis, partly on the rate at which existing lipoproteins were freed of their previous load of neutral fat and thus became available to carry more fat.

Under certain circumstances lipoproteins evidently fail to dispose of the neutral fat. This results in the accumulation in serum first of soluble neutral fat, and when this approaches 20 mEq. per L., of insoluble neutral fat which in turn causes lactescence (4). It thus appears that lipoproteins have only a limited ability to accept neutral fat.

There are certain similarities between the subnatant layers of chyle and of lactescent serum. The constancy of the ceiling for neutral fat in the subnatant fluid of lactescent serum at about 20 mEq. per L. (4) and of chyle at about 7 mEq. per L. (Figure 1) suggests that in both instances the lipoproteins are saturated with neutral fat. The lower concentration of protein-bound neutral fat in chyle than in serum equally lactescent is probably related to the lower concentration of protein in chyle.

The formation of a soluble free fatty acid-albumin complex in post heparin plasma to which chyle was added (27) suggests that free fatty acids occur at some stage of lipoprotein formation. The failure to demonstrate free fatty acids in the subnatant layer of chyle in the present study does not rule out their transient appearance.

It is not known with certainty which of the various lipoproteins transports neutral fat in the soluble form. Ultracentrifugal (28) and electrophoretic (29) studies suggest that the beta lipoproteins characterized by flotation rates of S_f 5 to 15 serve this function. These lipoproteins remain in the clear subnatant fluid under the conditions of centrifugation used in the present study (28). The concept of the beta lipoprotein molecule as a

[2] Experiments now in progress indicate that the lipids of liver lymph are not present in great enough concentrations to account for the soluble lipoproteins of thoracic duct lymph.

stable complex designed to transport a highly labile component, neutral fat, might explain the presence in serum of beta lipoproteins which are heterogeneous with respect to their flotation rates, that is, which contain varying loads of low-density neutral fat, but are antigenetically homogeneous (30). It might also explain the variability of serum neutral fat which is all the more striking because of the constancy of the other lipids in serum.

SUMMARY

1. Chylous fluid was obtained from non-fasting patients with external thoracic duct fistulae or chylothoraces. The chyle was universally lactescent. Neutral fat was the chief component, but cholesterol and phospholipid occurred almost en- varying concentrations. All lipid components were present largely in the emulsified state, and were removed by ultracentrifugation.

2. Chyle obtained from two fasting dogs with thoracic duct fistulae was only slightly lactescent and contained relatively little neutral fat. The cholesterol and phospholipid occurred almost entirely in the subnatant layer after centrifugation.

3. It appears that a fraction of newly formed, water-soluble lipoproteins are initially delivered to the thoracic duct, probably by way of the liver lymph. In the non-fasting state these lipoproteins are much richer in neutral fat than are the serum lipoproteins. This discrepancy is ascribed to the rapid removal of neutral fat from the lipoprotein molecule by the peripheral tissues.

4. The data support the concept of a lipoprotein molecule which is a stable protein-cholesterol-phospholipid complex designed to transport a highly labile, rapidly removed component, neutral fat.

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STUDIES OF THE COMPOSITION OF SECONDARY AMYLOID[1]

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As part of a study of the pathogenesis of secondary amyloidosis it became apparent that more precise knowledge of the components of amyloid substance would be necessary to determine the sources from which these components come and the reasons for their appearance.

Although previous studies have indicated that amyloid substance is predominantly protein (1, 2), with a lesser amount of carbohydrate (3, 4), the exact amount and nature of the carbohydrate moiety has not been defined. One reason for this is that amyloid, because of its relative insolubility, is difficult to extract from tissues. Those extracts which have been obtained yielded material which may have been quite different from amyloid in the natural state. Furthermore, it is difficult to identify amyloid in a given fraction since its only identifying characteristics, its affinity for Congo-red, metachromasia, and site of deposition, are no longer present.

The finding of an almost pure amyloid deposit at post mortem examination in a patient presented an excellent opportunity to determine the chemical composition of amyloid, unaltered by extraction procedures, and to obtain quantitative information on some of its components. In addition, similar studies were made on other tissues with and without amyloid to test the correlation between the concentrations of some of the components and the concentration of amyloid as estimated from histological examination.

MATERIALS AND METHODS

Materials

The relatively pure amyloid deposit was found in the liver of a 27-year-old white female dying of longstanding pulmonary tuberculosis and secondary amyloidosis of the liver, spleen, and kidneys. This lemon-sized tumor-like mass was firm and waxy in appearance and on histological examination appeared to consist almost entirely of amyloid substance. This substance exhibited the following histochemical staining properties: It stained slightly eosinophilic with hemotoxylin and eosin, exhibited metachromasia with methyl violet, had a strong affinity for Congo-red, a positive reaction with periodic acid-Schiff's reagent, and stained yellow with Verhoeff's elastic tissue stain with Van Giesen's counterstain. Schultz's stain for cholesterol, the Sudan III stain for fat, Baker's acid hematin stain for phospholipins and Feulgen's stain for desoxyribose nucleic acid were negative. Aside from the amyloid, some connective tissue and liver cell nuclei were seen. It was estimated that about 90 per cent of the tissue was amyloid substance.

The other tissues included in this study are listed in Table I, with their sources, and the approximate amount of amyloid present as judged from histological examination. All of the amyloid specimens represented secondary amyloid. The tissue labeled "BC" was the liver in which the amyloid deposit was found.

Methods

1. *Water content:* As soon as possible after obtaining the tissues, the water content was determined in duplicate by drying approximately 300 mgm. of tissue in vacuum over P_2O_5 at 110° C. overnight. All tissues were then placed in sealed bottles and deep frozen and specimens for analysis were cut without thawing. The tissues used in chemical studies were dried in vacuum at 110° C., and then pulverized. At the same time that portions of this powdered sample were removed for analysis, the water content of an aliquot was determined.

2. *Nitrogen:* Nitrogen determinations were done in quadruplicate by a micro Kjeldahl method using $CuSO_4$ and selenium as catalysts. The digestion was carried out for six hours. Water blanks, standard $(NH_4)_2SO_4$ and a purified gelatin standard[3] (nitrogen content of 18.3 per cent) were treated in the same fashion as the sample.

3. *Hexosamine:* The hexosamine content of the tissue was determined by the Boas modification of the Elson and Morgan reaction (5). The hexosamines and amino acids were separated from the neutral sugars and uronic acids by passing the acid hydrolysate of the tissue through a Dowex 50 ion exchange column. The neutral sugars and uronic acids, not being adsorbed by the resin, were obtained in the water wash; the hexosamines were adsorbed by the resin and finally eluted by 2 N HCl. The

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[2] Public Health Service Postdoctoral Research Fellow of the National Institute of Arthritis and Metabolic Diseases.

[3] Obtained from Dr. Jerome Gross.

TABLE I

Analyses of liver tissues with and without amyloid from both humans and horses

Tissue	Primary disease	Estimated* % amyloid	% H_2O	% of dry weight‡		
				Neutral sugar†	Hexosamine	Uronic acid
Amyloid deposit§	Tuberculosis	90	82.8	1.86	1.55	.60(2)
Human amyloid liver						
Zi	Rheumatoid arthritis	60	76.2	1.17(3)	.60(8)	.17(1)
Ad	Rheumatoid arthritis	50	80.0	1.57(3)	.96(4)	.28(1)
BC§	Tuberculosis	45	77.1	2.48(1)	.62(2)	
Human non-amyloid liver						
We	Hypertension	0	74.0	.92(2)	.25(6)	
Fr	Rheumatoid arthritis	0	75.1	1.20(3)	.32(2)	
To	Myocardial infarction	0	64.3	2.8 (1)	.22(1)	.23(1)
Ta	Cirrhosis of the liver	0	81.4		.36(1)	
Gi	Sudden death (cause undetermined)	0	74.1		.21(1)	
Pe	Myocardial infarction	0	77.4		.27(1)	
Hi	Carcinoma of ovary	0	64.9		.13(1)	
Horse amyloid liver						
By	Tetanus immunization	40	75.0	5.8 (1)	.54(3)	.45(1)
Re	Tetanus immunization	25	69.0	.75(2)	.35(2)	.15(2)
Bx	Tetanus immunization	20	78.8		.83(1)	
Horse non-amyloid liver						
Ka	Normal horse	0	69.5	4.9 (1)	.19(1)	.28(1)
Wh	Tetanus immunization	0	71.7	6.0 (1)	.25(1)	.25(1)
Em	Tetanus immunization	0	70.7	9.7 (2)	.28(2)	.43(2)
Le No. 1	Botulinus immunization	0	70.4	5.1 (2)	.35(2)	.33(2)
Le No. 2	Tetanus immunization	0	73.9	13.5 (2)	.35(2)	.56(2)

* The figures represent the mean of several observers' estimations of the amyloid concentrations of microscopic sections.

† Expressed as equal amounts of glucose and galactose.

‡ The numbers in parentheses represent the number of determinations made for each mean value. The hexosamine values were determined from samples hydrolyzed for 2 to 16 hours, whereas the neutral sugar and uronic acid values were determined from samples hydrolyzed for 2 to 8 hours. In each case one 8-hour hydrolysis was performed, and when only one determination was made, the hydrolysis period was 8 hours. The values for the amyloid deposit, however, are the best values obtained with varying hydrolysis times (hydrolysis time for the hexosamine, 12 hours; for the neutral sugar, 2 hours; for the uronic acid, 2 hours). 2 N HCl was used for hydrolysis in each case.

§ The tissue "BC" is the liver in which the deposit was found.

hexosamine content was then measured by the Elson and Morgan reaction. Optimal hydrolysis conditions of the tissue with hydrochloric acid were established by varying both the normality of the acid and the time of hydrolysis (up to 6 N and 24 hours, respectively). Glucosamine hydrochloride and purified ovomucoid [4] (12 per cent glucosamine) were used as standards.

4. *Neutral sugars:* The neutral sugars obtained by the method outlined in the preceding paragraph were determined by the anthrone method of Loewus (6). As the color intensity of anthrone-sugar complexes varies with the sugar, the neutral sugar content must be expressed in terms of those sugars present in the sample. Inasmuch as the amyloid deposit contained approximately equal amounts of glucose and galactose, the per cent of neutral sugar of all the tissues in this study was calculated on this basis (as the non-amyloid tissues contained more glucose than galactose, the true values for these tissues is slightly less than indicated). Optimal hydrolysis conditions with HCl were again determined by varying both normality and time of hydrolysis. This anthrone method was also applied directly to the dried, powdered, unhydrolyzed amyloid tissue (7). Glucose, galactose, mannose, and ovomucoid (10 per cent mannose) were used as standards.

5. *Uronic acids:* The uronic acid content of the water wash obtained above was determined by the napthoresorcinol method of Fishman, and his co-workers (8) recently modified as follows: To 3 cc. of the sample were added 2 cc. of .4 per cent naphthoresorcinol, and 2 cc. of 18 N H_2SO_4. This was heated in an unstoppered test tube for one and one-half hours on a steam bath. The optical density of the toluene extracted pigment was read at 575 mμ. The optical hydrolysis conditions of the tissue with HCl were again determined by varying the normality and time of hydrolysis. The naphthoresorcinol method was applied as well to the dry, powdered, unhydrolyzed amyloid tissue. Glucuronolactone and galacturonic acid were used as standards.

[4] Obtained from the Worthington Chemical Corporation.

6. *Amino acids and inorganic elements:* Quantitative determinations of hydroxyproline (9), tyrosine (10), glycine (11), ash, total phosphorus[5] and total sulfur,[5] were made by standard methods.

7. *Chromatography:* The identification of the carbohydrate components was carried out by paper chromatographic methods. For the neutral sugar identification samples of approximately 50 mgm. were hydrolyzed with both HCl and H_2SO_4 in varying strengths and times. The optimal hydrolysis conditions were: hydrolysis with 4 N HCl for two hours at 100° C., removal of the excess HCl with $AgCO_3$, then passage of the filtrate through a Dowex 50 ion exchange column. The water wash was evaporated to dryness under nitrogen on a steam bath and dissolved in small amounts of 50 per cent ethanol in water for application to the paper. Three separate techniques and solvent systems were used: descending partition with butanol-ethanol-water (4:1:1), for 44 hours, and multiple ascending development (12) with butanol-pyridine-water (6:4:3), and with ethylacetate-acetic acid-water (3:1:3). Both ammoniacal silver nitrate and analine phthalate were used to develop the chromatograms.

Preparation of the sample for hexosamine chromatography was identical with that for neutral sugars except that hydrolysis was longer (six hours) and the hexosamine was eluted from the resin column with 2 N HCl. The sample was evaporated to dryness on a steam bath under nitrogen. The paper chromatographic procedure consisted of partition of the hexosamines in butanol-ethanol-water (4:1:1), conversion of the hexosamines to their characteristic pentoses with ninhydrin, and then partition in a second dimension, as described by Stoffyn and Jeanloz (13). Some trailing of the hexosamines occurred during the first partition in this solvent system, but should not prove objectionable; for there are no known substances other than hexosamines which have a very low R_f in this solvent system, that may be converted by ninhydrin into pentoses which have a relatively high R_f in the same solvent system.

The relative amounts of the monosaccharides identified by these chromatograms were estimated directly with an A. H. Thomas Company Densigraph. Standard monosaccharides were prepared in the same fashion as the samples for identification of the unknowns and for densimetric comparison.

RESULTS

The water concentration of the amyloid deposit was 82.8 per cent. This figure corresponded with a water concentration of 77.1 per cent for the liver in which the specimen was found, suggesting a hydrophilic nature of this substance. Moreover, two of the three amyloid livers from horses exhibited water concentrations above the range of values found for the non-amyloid horse livers. This same trend appeared in the water concentration of the amyloid livers from humans if compared with the value of 68 per cent for the water concentration of liver from normal humans (14). No suitable normal human controls were available for this study and so the values shown for the non-amyloid livers from patients dying from a variety of acute and chronic diseases were spread over a wide range.

The nitrogen concentration of the amyloid deposit was 14.20 per cent of its dry weight as compared with values of 12.10 per cent and 12.45 per cent for two non-amyloid livers from humans, "We" and "Fr," respectively. The nitrogen concentration of the amyloid liver, "Zi," was 13.50 per cent.

The neutral sugar concentration of the amyloid deposit was 1.86 per cent of its dry weight after an optimum hydrolysis and separation on the resin column, and 2.21 per cent as determined on the unhydrolyzed liver tissue. These values are in rough agreement and are evidence that hydrolysis and recovery of the neutral sugars was adequate. Optimal hydrolysis of the amyloid deposit was found to occur with 2 N HCl for two hours at 100° C. Stronger acid or longer periods of hydrolysis caused considerable destruction of the sugars. All the other tissues were similarly hydrolyzed with 2 N HCl, although there was little difference in the yield for hydrolysis times between two and eight hours. Therefore, the neutral sugar values listed in Table I for the tissues other than the amyloid deposit, represent the means of all values with a hydrolysis time between two and eight hours. These values do not show significant differences between the amyloid and non-amyloid tissues. The high values for the non-amyloid horse livers probably reflect glycogen storage.

The hexosamine concentration of the amyloid deposit was 1.55 per cent of the dry weight. This optimum value was obtained after hydrolysis for 12 hours with 2 N HCl, although the yield with hydrolysis times at any interval between 2 and 16 hours did not differ significantly from this optimum value. The hexosamine values for the other tissues listed in Table I again demonstrate little difference when hydrolyzed for any interval between 2 and 16 hours, and therefore the

[5] The phosphorus and sulfur determinations were performed by Dr. Karl Ritter, Basel, Switzerland.

values expressed in this table represent a mean of determinations within this range. Because others (5), however, have suggested that the optimal hydrolysis for hexosamines in liver tissue occurs at eight hours, at least one determination for each tissue was done at this time, and if only one hydrolysis was performed, it was for eight hours. As can be seen in Table I, the hexosamine concentration of amyloid containing livers was not only slightly higher than that of non-amyloid livers but also proportional to the amount of amyloid present. The range of values for the non-amyloid livers agreed well with that reported by others (5) for normal humans.

The uronic acid concentration of the deposit, obtained in maximum yield by hydrolyzing with 2 N HCl for two hours, was .60 per cent. This value was in close agreement with the .57 per cent obtained by direct determination on the powdered unhydrolyzed amyloid tissue. There appears to be no significant difference between the values of the amyloid tissues and the non-amyloid tissues listed in Table I.

The total phosphorus concentration of the deposit was .03 per cent of dry weight (5.1 mgm. per cent of wet weight) as compared to .67 per cent (153 mgm. per cent wet weight) for the liver tissue "BC" in which the deposit was found. The total phosphorus concentration of a non-amyloid liver "We" was .84 per cent (219 mgm. per cent wet weight). The total sulfur concentration of the deposit was .86 per cent of the dry weight. The ash content was 3.2 per cent of the dry weight.

Chromatographic studies of the neutral sugar fraction of the amyloid deposit hydrolysate showed that approximately equal amounts of glucose and galactose were present (see Figure 1) and accounted for the major portion of the neutral sugars. This finding was consistent with three different solvent systems. In addition reducing substances corresponding to mannose, fucose, and arabinose were detected in small quantities in those chromatograms developed with ethyl acetate-acetic acid-water. Two other faint spots with low R_f values and one with a high value were noted but could not be further identified.

Chromatographic identification of the hexosamine components of the amyloid deposit revealed that both glucosamine and galactosamine were

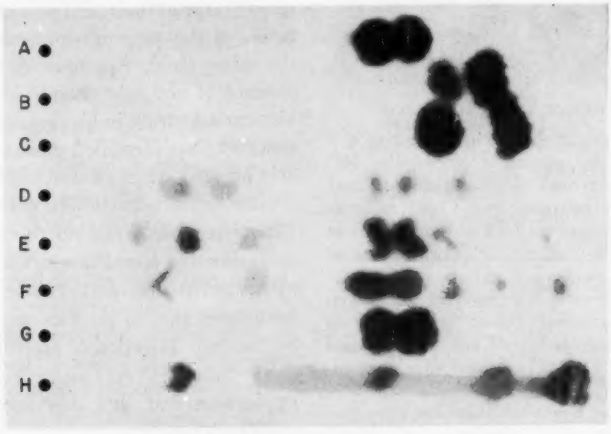

FIG. 1. CHROMATOGRAM FOR NEUTRAL SUGARS

Whatman No. 54 paper with 3 ascending developments, in butanol, pyridine and water (6:4:3) stained with ammoniacal silver nitrate. The spots, reading from left to right are: A. Standard galactose, glucose; B. Standard mannose, fucose; C. Standard arabinose, ribose; D. Deposit hydrolysate (5 mgm., 2 N HCl, 8 hrs.); E. Deposit hydrolysate (20 mgm., 4 N HCl, 6 hrs.); F. Deposit hydrolysate (20 mgm., 4 N HCl, 2 hrs.); G. Standard galactose, glucose; H. Standard glucuronic acid, galactose, N-acetyl glucosamine, glucuronolactone.

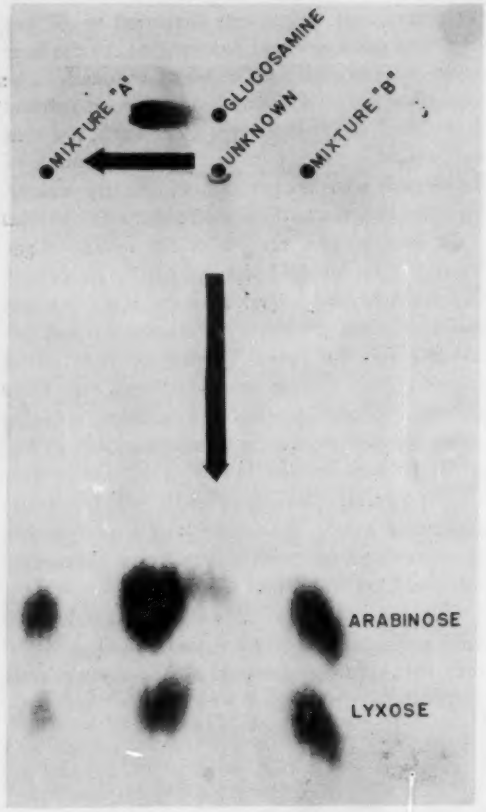

FIG. 2. CHROMATOGRAM FOR AMINOSUGARS

The unknown (20 mgm. of deposit hydrolyzed in 4 N HCl for 6 hours) and 100 gamma of glucosamine HCl developed 3 times (Top arrow) by an ascending method. After trimming off the reference spot (Top), mixture "A" (50 gamma of glucosamine HCl and 20 gamma of galactosamine HCl) and mixture "B" (100 gamma of glucosamine and 100 gamma of galactosamine HCl) were applied, the paper treated with ninhydrin and developed by a descending method (lower arrow) for 40 hours. Paper was Whatman No. 54 and solvents were butanol, ethanol, water (4:1:1), stained with ammoniacal silver nitrate.

The lower spots on the figure are arabinose from glucosamine and lyxose from galactosamine.

present in a molecular ratio of approximately 4 to 1 (see Figure 2).

The concentrations of a few amino acids as determined by colorimetric methods were: hydroxyproline 1.5 per cent; glycine 3.8 per cent; and tyrosine 4.1 per cent. The low hydroxyproline and glycine concentrations of this deposit indicate that collagen represented less than 10 per cent of the dry material.

DISCUSSION

Amyloid may be defined as a histologically homogeneous, amorphous material with characteristic sites of deposition and staining reactions. There yet remains a chemical analysis of the total tissue to establish whether this histologically homogeneous material is a single chemical complex, or whether it is a mixture of substances.

These data indicate that amyloid substance is protein combined with a small amount of carbohydrate. The histochemical characteristics of amyloid, *i.e.*, the metachromasia and periodic acid-Schiff reactivity, have long suggested that carbohydrates are present in the structure of amyloid. Nevertheless, the exact nature and amount of these carbohydrate components have been poorly defined. Krawkow (1) and later Hass (4) extracted from amyloid bearing organs small amounts of a substance which they believed to be similar to chondroitin sulfuric acid. Furthermore, Ehrström (3) noted that a mixture of chondroitin sulfuric acid and blood serum assumed a few of the staining properties of amyloid. On the other hand, Eppinger (2) comments on the absence of not only chondroitin sulfuric acid, but also carbohydrate in his preparation. Meyer (15), however, has identified glucosamine and a uronic acid in an extract of amyloid and felt that these constituted a heparin-like polysaccharide. Faber (16) has commented on the fact that more glucosamine was found in amyloid bearing liver than in a normal one, and Tolone and Pansini (17) have been able to produce amyloidosis in rabbits by the daily injection of glucosamine. Finally the name "amyloid" has unfortunately ingrained the impression that this substance is predominantly carbohydrate.

The results of this study indicate that carbohydrate could scarcely contribute more than 4 per cent of the solid material. Both glucosamine and galactosamine were identified chromatographically in the ratio of 4 to 1, indicating that chondroitin sulfuric acid is not a major carbohydrate component. Furthermore, the hexosamine

concentrations found in several amyloid containing livers from both human and horses were roughly proportional to the amyloid concentration as judged from histological examination. This serves to underscore the importance of this component in amyloid. Although other investigators have isolated from amyloid small amounts of polysaccharide, containing hexosamine, they have not accounted for as large an amount of hexosamine as observed in this study. Furthermore, the finding of bound neutral sugar in addition to the other carbohydrates indicates that amyloid substance, though homogeneous histologically, is probably a mixture of protein, glycoprotein and polysaccharide; for no carbohydrate complex has to our knowledge been isolated that contains the three carbohydrates—amino sugar, neutral sugar, and uronic acid.

The low total phosphorus concentration of the amyloid deposit was far below that found in the liver tissue surrounding the tumor, and was below that which would be expected were nucleic acid present in the same concentration in amyloid as in normal liver (normal human liver contains 85 mgm. of nucleo protein phosphorus per 100 gm. of wet weight, [18]). One would infer, therefore, that amyloid is probably not formed by mere coalescence of cytoplasmic and nuclear proteins of the liver into a homogeneous mass.

SUMMARY

The chemical composition of a relatively pure amyloid deposit obtained from the liver of a patient dying of tuberculosis and secondary amyloidosis has been studied.

1. Data are presented that indicate that it was predominantly a hydrophilic protein.

2. There were approximately 2 per cent of neutral sugars of which the main constituents were equal amounts of glucose and galactose.

3. The hexosamine concentration was 1.5 per cent. Both glucosamine and galactosamine were identified by chromatographic methods with the former predominating.

4. About .6 per cent of uronic acid was also found.

5. The total phosphorus concentration of this deposit was much less than that found in the surrounding liver tissue.

6. Observations of other human and horse livers both with and without amyloid, suggest that the hexosamine concentration and amyloid concentration were related.

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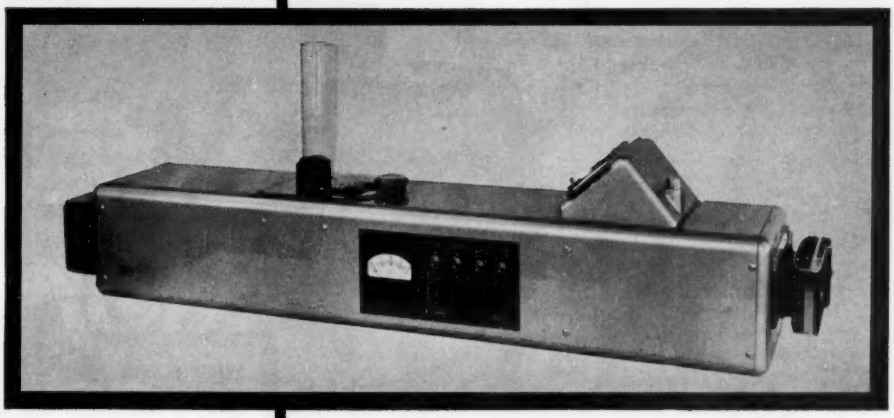

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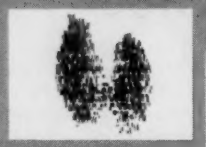

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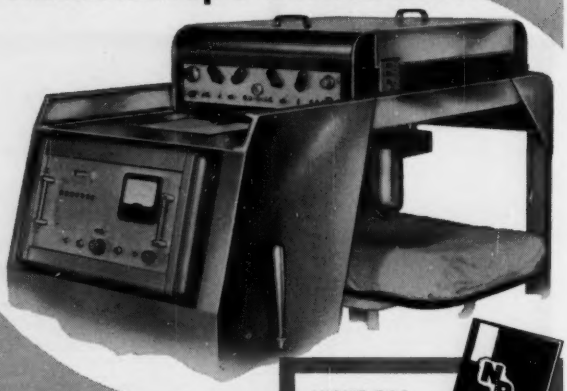

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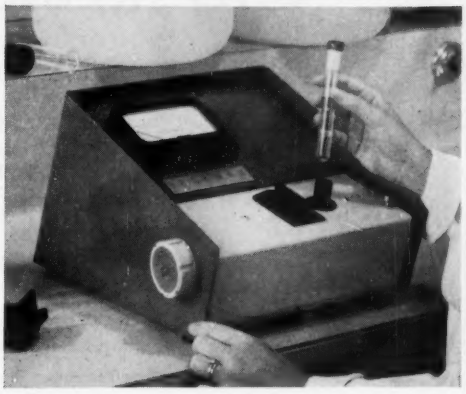

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